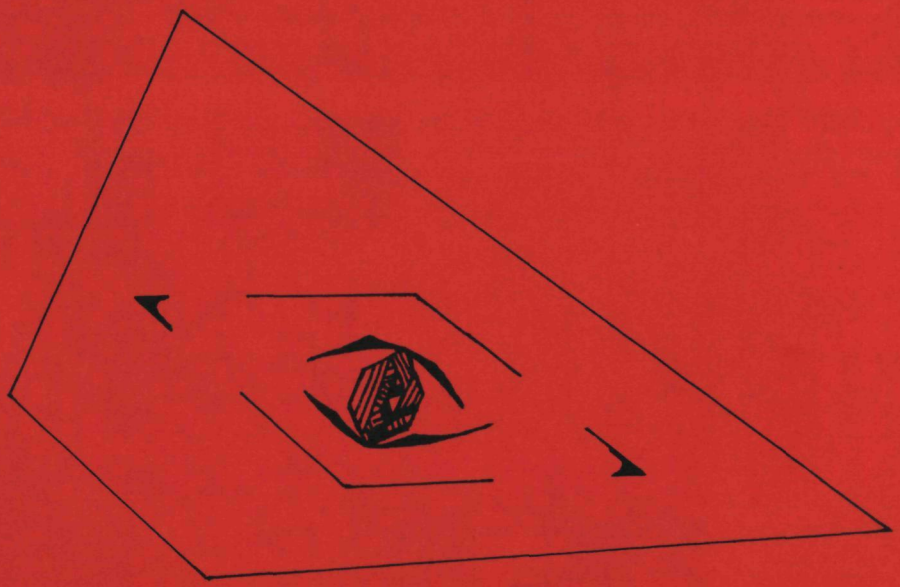
BOVINE RETINAL PIGMENT EPITHELIUM

STUDIED IN VITRO



A.M.M. Timmers

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ISBN-90-9001567-1

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PROEFSCHRIFT

ter verkrijging van de graad van
doctor in de wiskunde en natuurwetenschappen
aan de Katholieke Universiteit te Nijmegen
op gezag van de Rector Magnificus
Prof. Dr. B.M.F. van Iersel
volgens besluit van het College van Decanen
in het openbaar te verdedigen op woensdag
25 februari 1987 des namiddags
te 1.30 precies

door
Adrian Martin Mary Timmers
geboren te Hamilton (Canada)

Druk: Quick Service Drukkerij Enschede

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Cover artistics: Gerrie M.W. van den Crommenacker

The investigations described in this thesis were carried out at the Department of Biochemistry, Center of Eye Research, University of Nijmegen, Nijmegen. Financial support was obtained from the U.O.P. foundation, University of Nijmegen.

Dankwoord.

Iedereen die heeft bijgedragen aan het experimentele en redactionele deel van dit proefschrift wil ik op deze pagina bedanken. Zonder anderen daarbij te kort te willen doen, zal ik enkelen van hen met name vermelden.

Diny van Groningen-Luyben voor haar kritische inbreng en experimentele vaardigheden die van grote betekenis waren voor het onderzoek, Rob Rutten voor de morfologische en filosofische benadering en alle andere medewerkers voor de leuke periode en prettige samenwerking.

Prof. Dr. Edward A. Dratz who, in his dynamic and stimulating way, initiated the development of the perfusion technique which resulted in a joint publication.

Dr. R. De Abreu and J. Baal voor hun uitstekende energy charge analyses.

De doctoraal studenten en Olan stagiaires (alfabetisch): Mart Beelen, Petra v.d. Broek, Marten Dooper, Elly Franken, Gonneke Gille, Hans de Jonge, Jolanda Laurensen, Laurent Nelissen, Marjolein Peeters en Ruud van Roosmalen.

De afdeling Pathologische Anatomie voor hun assistentie in het voorbereiden van microscopische preparaten. De afdeling Submicroscopische Morfologie voor het beschikbaar stellen van apparatuur en Prof. Dr. A.M. Stadhouders voor de getoonde belangstelling en zinvolle discussies.

De medewerkers van de afdeling Medische Illustratie tekenden voor het maken van grafieken en afdrukken van foto's.

De vleespreparateurs van het slachthuis te Nijmegen voor het bijbrengen van de kunst om runderogen met 'lange steel' te verkrijgen en het dulden van mijn aanwezigheid aan de lopende band.

Verder gaat mijn dank uit naar de universiteit voor het beschikbaar stellen van energie, zodat ten alle tijde koffie kon worden gezet.

Als laatste, maar natuurlijk niet de minste, mijn ouders voor hun nimmer aflatende ondersteuning en belangstelling in mijn werk en mijn 'zijn'. Verder familie en vrienden en in het bijzonder Mary.

Parts of this thesis have already been published or will be:

Ad M.M. Timmers, Edward A. Dratz, Willem J. de Grip, and Frans J.M. Daemen
A new isolation technique for retinal pigment epithelium
Invest. Ophthal. Vis. Sci. 25: 1013-1018 (1984)

Ad M.M. Timmers, Diny A.H.M. van Groningen-Luyben, Frans J.M. Daemen, and Willem J. de Grip
A rapid versatile microassay for cellular retinol binding protein using Lipidex-1000 microcolumns
J. Lipid Res. 27: 979-987 (1986)

Ad M.M. Timmers, Willem J. de Grip, and Frans J.M. Daemen
A retinal pigment epithelium viewpoint on vitamin A metabolism
In: Proceedings on Retinal Proteins
Ed: N.G. Abdulaev and Y.A. Ovchinnikov
VNU Science Press, Utrecht 1986 (in the press)

Ad M.M. Timmers, Diny A.H.M. van Groningen-Luyben, and Willem J. de Grip
Metabolism of all-trans retinol in isolated bovine retinal pigment epithelium
Submitted for publication

ABBREVIATIONS

ADP	: adenosine diphosphate
AMP	: adenosine monophosphate
ATP	: adenosine triphosphate
cRABP	: cellular retinoic acid binding protein
cRAlBP	: cellular 11-cis retinoid binding protein
cRBP	: cellular retinol binding protein
DAPI	: 4',6-diamidino-2-phenylindole.2HCl
DNA	: deoxyribonucleic acid
EDTA	: ethylene diaminetetraacetate
FITC	: fluorescein isothiocyanate
HEPES	: N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
HPLC	: high performance liquid chromatography
IRBP	: interphotoreceptor retinoid binding protein
IPM	: interphotoreceptor matrix
kD	: kilo Dalton
Kd	: dissociation constant
Km	: Michaelis-Menten constant
LDH	: lactate dehydrogenase
M_r	: relative molecular mass
ROS	: rod outer segments
RPE	: retinal pigment epithelium
SD	: standard deviation
SDS-PAGE	: sodium dodecyl sulphate-poly acrylamide gel electrophoresis
SE	: standard error
sRBP	: serum retinol binding protein

CONTENTS.

Chapter 1

RETINAL PIGMENT EPITHELIUM CELLS AND VISION:

AN INTIMATE RELATIONSHIP.

1.1. THE EYE.

1.1.1. INTRODUCTION.

Light plays an indispensable role in nature. Many species of the animal kingdom as well as of the plant kingdom contain structures specialized for light detection in order to be able to perform functions like photosynthesis, photoperiodism and vision. The common characteristic of all light receptors is their constitution of pigments. As far as known all these pigments are chromoproteins or protein complexes, of which the 'prosthetic' group contains conjugated polyene systems.

Vision, light reception, is restricted to the animal kingdom. The light detectory organ is the eye, which occurs in a variety of forms. All types of eyes contain in their photoreceptor cells one or more visual pigments, which throughout the animal kingdom appear to be a set of evolutionary related proteins, opsins, covalently linked to a 11-cis vitamin A derivative as chromophore. Between absorption of light in the eye and its perception in the brain, the photoreceptor cells are the actual site of visual excitation: the translation of light signals into neuronal activity.

It would go far beyond the scope of thesis to consider all aspects of vision. I will restrict myself to the vertebrate eye. In fig 1.1 the basic design of the vertebrate eye is shown. Sclera and cornea form the outer support. Light entering the eye is refracted by the cornea, lens to form an image on the light sensitive layer, the retina. The retinal pigment epithelium (RPE) is located distally to the retina and proximally to the highly vascularised choroid.

1.1.2. THE RETINA.

The retina consists of several cell types (fig. 1.2), which all participate in the primary neuronal conduction of the light signal: ganglion, bipolar and horizontal cells and most distally the photoreceptor cells, where the actual photon absorption occurs. The Müller cells, neuroglia cells extending from the inner to the external limiting membrane, provide mechanical support to the retina. The RPE cells border the photoreceptor cells distally.

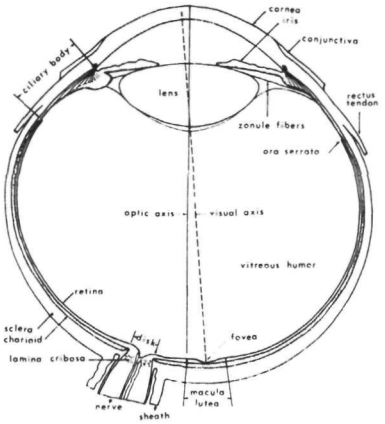


Fig. 1.1
Schematic diagram of a cross section of the vertebrate eye (human).

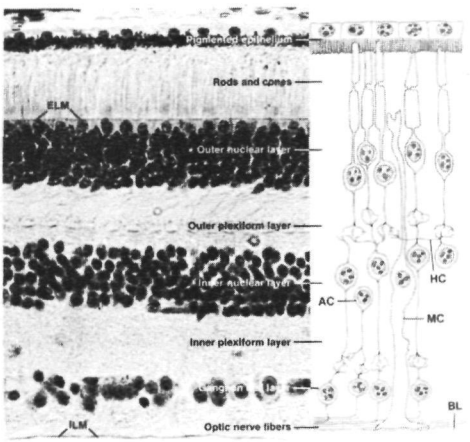


Fig. 1.2
Micrograph and schematic diagram of the vertebrate retina.
ELM represents: the external limiting membrane, ILM: the inner limiting membrane, BL: the basal lamina, HC: horizontal cells, AC: amacrine cells and MC: Muller cell.

1.1.3. ANATOMY OF THE PHOTORECEPTOR CELLS.

Both photoreceptor cells, rods and cones, can be subdivided in two structural elements: the inner and the outer segment (fig. 1.3). The inner segment, forming synaptical junctions with the bipolar and horizontal cells at its proximal end, contains the normal cell organelles: nucleus, mitochondria, (rough) endoplasmic reticulum, the Golgi apparatus and the lysosomes. The general metabolic cell processes (cellular regulation, protein synthesis, energy metabolism) are carried out in this part of the photoreceptor cell. The outer segments contain a stack of membranes

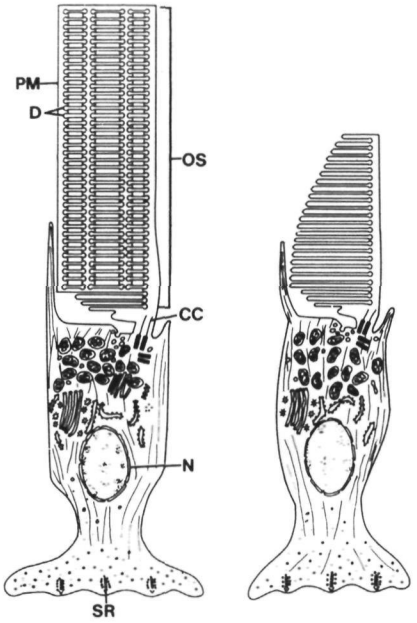


Fig. 1.3
Schematic diagram of the rod (left) and cone (right) photoreceptors.
The outer segment (OS) contains a pile of discs (D) in which the visual pigment is embedded. The discs are surrounded by a plasma membrane from which the discs develop through evagination. The outer segment is linked to the inner segment by a connecting cilium (CC). The inner segment contains the normal cell organelles. At its base, the photoreceptor cell conducts the light initiated signal through the synaptic terminal (which contains numerous synaptic ribbons (SR).

(discs), which is surrounded by the plasma membrane and in which the light sensitive protein is embedded.

Dependent on the type of photoreceptor cell, two types of outer segments can be distinguished: the rod outer segment (ROS) and the cone outer segment (COS), both are connected to the inner segment by a cilium. The major structural difference between the COS and ROS, in addition to their morphology, is manifested by the discs: the ROS contain closed separated discs, enveloped by a plasma membrane, while the COS discs remain continuous with the plasma membrane and open to the extracellular space. Rods and cones are complementary to each other in their function as light receptor. Rods function in twilight (scotopic vision), while cones have a much higher signal treshold for light and function primarily under daylight conditions (photopic vision). Cones further mediate color vision. To serve conciseness, further discussion will be focused on the rod outer segments.

1.2. BIOCHEMISTRY OF LIGHT DETECTION.

1.2.1. THE VISUAL PIGMENT.

In rod outer segments the light sensitive element is the visual pigment rhodopsin: a complex of a vitamin A derivative and a protein opsin (fig 1.4). Opsin is a glycosylated polypeptide of 348 amino acids (Ovchinnikov et al., 1982; Hargrave et al., 1983) with a molecular weight of 39 kD and probably a seven-helix arrangement through the disc membrane. About 50% of the protein mass is embedded in the lipid bilayer. The latter is exceptional in its high (over 50%) content of polyunsaturated fatty acids (Borggreven et al., 1970; Anderson and Maude, 1970; Miljanich et al., 1979; Drenthe et al., 1981). The C-terminal end of the protein is located at the cytoplasmic surface of the disc membrane, the N-terminal end, which contains two carbohydrate moieties, is located intradiscally. The actual light sensitivity is generated by combining 11-cis retinaldehyde to the apoprotein, opsin. Covalent binding occurs via a Schiff-base linkage to lysine residu 296 (de Grip et al., 1973; Wang et al., 1980). The holoprotein, rhodopsin,

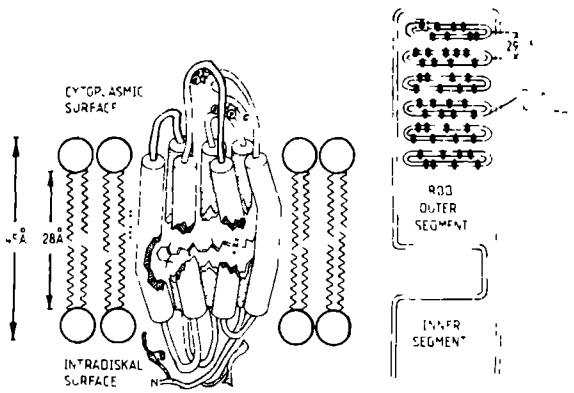


Fig. 1.4
Schematic diagram of the rod outer segment and an expanded view of rhodopsin.
Rhodopin is embedded in the membrane by a seven-helix arrangement. the C-terminal part is located at the cytoplasmic side and intradiscally the N-terminus with two carbohydrate moieties. The chromophoric group is completely located in the hydrophobic part of the protein.

has an absorption maximum around 500 nm for most mammals. Due to minor modifications in the apoprotein, changes in the λ max can be achieved, which probably accounts for most species differences.

1.2.2. SIGNAL TRANSDUCTION.

When a photon with appropriate energy activates rhodopsin, a transient sequence of spectrally different intermediates is observed (Abrahamson, 1973). The 11-cis retinaldehyde isomerizes to all-trans (Wald, 1968), which causes tension in the protein and results in small conformational changes. The all-trans retinaldehyde will finally leave the protein (photolysis). The metarhodopsin stage II (λ max: 380 nm) is the active intermediate in signal transduction (Bennet et al., 1982; Emeis et al., 1982), which is initiated by the binding of a G-protein (transducin), probably at specifically exposed 'signal-sites' at the cytoplasmic side of rhodopsin (Kühn, 1984; Stryer, 1985). The G-protein, which consists of the 3 subunits: α (40 kD), β (38 kD) and γ (8 kD) exchanges GTP for GDP upon binding to the light activated rhodopsin. The GTP binding α-unit then leaves the complex and turns on a phosphodiesterase (PDE) through inactivation of the PDE inhibitor. The subsequent increased hydrolysis of c-GMP results in a local drop in c-GMP concentration. Since c-GMP effectuates longer 'open-times' of a plasmamembrane located Na^+-channel, a decrease in c-GMP concentration will lower the Na^+-permeability of the plasma membrane leading to a hyperpolarization of the plasma membrane (Miller, 1983; Knowles, 1984; Korenbrot, 1985). The signal is conducted via the membrane of the photoreceptor cell synaps, modulated and processed by intermediate neurons and carried via the optic nerve to the brain. This light detection system is, in principle, able to detect a single photon (Bouman and Van de Velden, 1947), which illustrates its huge amplification potential. Down regulation of the transduction system is effectuated by (i) re-activation of the PDE inhibitor due to the hydrolysis of the GTP bound to the transducin α-unit, (ii) phosphorylation of rhodopsin by a rhodopsin kinase (Kühn, 1984; Wilden et al., 1986; Pfister et al., 1985) followed by binding of arrestin (48 K protein or S-antigen), and (iii) decay of activated rhodopsin into the apoprotein opsin. The system then has to be re-activated by combining opsin with 11-cis retinaldehyde.

1.2.3. MAINTENANCE OF THE LIGHT SENSITIVITY.

Continuous light detection requires regeneration of the apoprotein with the chromophoric group into an active visual pigment, as well as de novo biosynthesis of rhodopsin.

1.2.3.1. Regeneration of rhodopsin.

The photolysis of rhodopsin leads to opsin and all-trans retinaldehyde. To regain light sensitivity, regeneration has to take place. In vitro, this can be accomplished by merely adding 11-cis retinaldehyde to opsin, in vivo, the final step must be the same. That part of the metabolic pathways of vitamin A, which are intimately related to the bleaching and regeneration of rhodopsin, is called the visual cycle. Both the retina and the RPE cells play a role in this process, which will be discussed in more detail in section 1.4.3.

1.2.3.1. Biosynthesis of rhodopsin.

Next to re-use of opsin, continuous biosynthesis of opsin is carried out by the inner segment of the photoreceptor cells, which effectuates a complete renewal of the entire outer segment in approximately 10 days in homoiothermic animals (40-50 days in poikilothermic animals) (Young, 1967, 1971; Hall et al., 1969). The opsin in statu nascendi is transported to the basal part of the outer segments (cf Papermaster and Schneider, 1982) where the assembly of the discs takes place through evagination of the outer segment plasma membrane (Steinberg et al., 1980). At the distal end, in overall pace with the supply, disc packets are phagocytized by the RPE cells. This phenomenon will be considered in detail in a later section (1.5).

1.3. THE RETINAL PIGMENT EPITHELIUM.

The RPE cells are juxtaposed between the retina and the choroid, with the apical side facing the outer segments. The basal membrane, cemented on the basement membrane, is separated from the choroid by Bruchs membrane. Prior to exposing the role of the RPE in retinal processes, like the visual cycle and phagocytosis, into more detail, some general background information will be provided as foundation.

1.3.1. ANATOMY OF THE RETINAL PIGMENT EPITHELIUM.

The RPE cells form a monolayer of cells extending from the optic disc to the ora serrata and have a typical hexagonal epithelial morphology. The cells measure about 10 - 14 µm in height and 15 - 25 µm in diameter and are interconnected by elaborate junctional complexes. They show a characteristic polarized structure. A cross section (fig 1.5) reveals socalled basal infoldings at the basal side, a cytoplasm with a high density of membraneous structures ((rough) endoplasmic reticulum, Golgi apparatus), nucleus, mitochondria, lysosomes and phagosomes and an apical side containing melanosomes and extending into microvilli, which surround the outer segments of the photoreceptor cells (cf Rodieck, 1973).

1.3.2. THE INTERRELATION BETWEEN THE RETINAL PIGMENT EPITHELIUM AND THE RETINA.

Ontogenetically, the retina and RPE cells are closely related. The neuroectoderm of the proencephalon evaginates into the optic

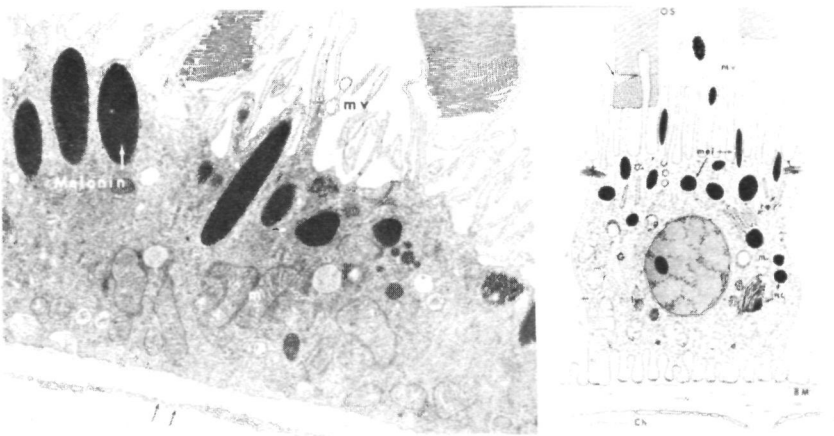


Fig. 1.5
Transmission electron micrograph and schematic diagram of retinal pigment epithelium.
The basal part, with basal infoldings, is cemented to Bruchs membrane which separates RPE cells from the choroidal vasculature. In addition to normal cell organelles, melanin granules(m) are present. At the apical part, the membraneous extensions, microvilli (mv), enfold the tips of the outer segment of the photoreceptor. Note the high density of membraneous structures in the RPE cell.

stalk forming distally the optic vesicle (which on their turn induce the formation of the lens placodes). The external layer and internal layer, resulting from invagination of the optic vesicle, differentiate into the RPE and retina respectively. The RPE induces surrounding mesenchymal tissue to develop choroid and sclera (Hoar, 1982; cf Rodieck, 1973).

From a physical point of view, the retina and RPE maintain an intimate relationship: the membraneous extensions (microvilli) of the RPE enfold the outer segments. During development of the retina, simultaneous with the differentiation of the outer segments, the interphotoreceptor matrix (IPM) develops. This is a gel like matrix of glycoproteins and glycosaminoglycans (GAGs) (Zimmerman, 1958; Bach and Berman, 1971) and is probably involved in retinal attachment (De Guillebon and Zauberman, 1972; Zauberman, 1979). Further it allows easy exchange of metabolites between RPE and retina.

1.3.3. FUNCTIONS OF THE RETINAL PIGMENT EPITHELIUM.

The retinal pigment epithelium fulfills a number of functions of vital importance for the retina. Some major ones are summarized below:

A. The RPE cells specifically exchange substances eg vitamin A, amino acids (Steinberg and Miller, 1979; Pautler and Tengerdy, 1986), ions (Hughes et al., 1984; Miller and Farber, 1984; Immel and Steinberg, 1985) to and from the retina. Metaphorically, the RPE acts as a compiler of signals from the environment (blood circulation) to outer segments of the retina.

B. The RPE cells maintain the constant length of the outer segments. The ongoing de novo synthesis, which tend to elongate the outer segments continuously (Droz, 1963; Young, 1967), is compensated by the phagocytotic activity of the RPE cells. In RCS rats, the retinal pigment epithelium is defective in phagocytosis and the continuous elongation of outer segments with consequent buildup of membraneous debris between RPE and retina, eventually results in degeneration of the photoreceptor layer (Herron et al., 1969; Bok and Hall, 1971).

C. The RPE forms a major part of the blood-retina-barrier in addition to the retinal vasculature, which contains no

fenestrations and is even absent in some species. The barrier, in analogy with the blood-brain-barrier, allows the retinal tissues its specific physiological environment and protects them for several toxic compounds (Cunha-Vaz, 1980).

D. The RPE cells enhance visual acuity. Black melanine granules function as a light trap for stray light. In some species, part of the RPE cell layer does not contain this black pigment. This tapetum lucidum reflects light not absorbed by the retina which will produce a more blurred image but will considerably enhance light sensitivity.

E. The RPE cells participate in the synthesis of glycosaminoglycan (GAG) and glycoproteins, constituting the gel-like interphotoreceptor matrix, which fills the subretinal space (Berman, 1964; Berman and Bach, 1968; Tesosiere et al., 1971; Young, 1973). The photoreceptor cell layer also participates in the production of the interphotoreceptor matrix by secreting GAG (Ocumpaugh and Young, 1966) and the major glycoprotein: *interphotoreceptor retinoid binding protein*, IRBP (*Hollyfield* et al., 1985a; cf Bridges et al., 1986a).

F. The RPE cells' capacity to remove fluid from the subretinal space represents the major force for retina adhesion (Zauberman, 1979; Marmor et al., 1980).

1.3.4. SPECIAL ACTIVITIES OF THE RETINAL PIGMENT EPITHELIUM.

Whereas the oxygen consumption of the retina is higher than in most other tissues (Cohen and Noell, 1965), the activity of several oxidative enzymes is even higher in the RPE than in the retina (Pearse, 1961; Hanson, 1970). The activities of the RPE apparently are very energy demanding.

The biochemistry of RPE comprises a variety of specialistic metabolic activities. Here, melanin and lipofucsin will be briefly discussed. Separate sections will be dedicated to vitamin A metabolism and phagocytosis of rod outer segment tips.

Melanin.

The pigment granules, which originally provided the name of the retinal pigment epithelium, contain the brown-black pigment eumelanin. These granules are assembled early during ontogenetical development of the cell and undergo little modification and low

turnover during the rest of cell life. The basic pathways are the following: tyrosine is converted via dihydroxyphenylalanine into dopaquinone (Raper, 1928) by the copper containing tyrosinase (Vanneste and Zuberbuhler, 1974), which activity probably is restricted to the premelanosomes. Dopaquinone is further processed into a protein containing black precipitate: melanin. In RPE cells overlaying the tapetum lucidum, which do not contain pigment granules, melanin synthesis is blocked. Currently, there is no indication whether this regulatory block occurs at the level of the novo protein synthesis and whether extracellular factors are involved (cf Feeney-Burns, 1980).

Lipofucsin.

In addition to the melanin pigment granules, often autofluorescent granules are detected in RPE cells. In contrast to the melanin granules their number increases with age. The accumulation of this aging pigment, lipofucsin, is a result of phagolysosomal activity. Lipofucsin granules contain peroxidation products of fatty acids and lipids and their formation is enhanced in case of deficiency of antioxidants like vitamin E and selenium (Katz et al., 1982; cf Feeney-Burns, 1980).

1.4. RETINOIDS AND THE VISUAL CYCLE.

Some general aspects of retinoids, retinoid binding proteins and the current knowledge of the visual cycle will be discussed.

1.4.1. RETINOIDS.

1.4.1.1. Structure and nomenclature.

The retinoids are a class of compounds consisting of 4 isoprenoid units joined in a head to tail fashion representing a conjugated system of a long side chain with a β-ionone ring at one side and a variable end group at the other side (fig 1.6). Vitamin A represents a class of retinoids, excluding carotenoids, which exhibit the biological activity of retinol and include both naturally occurring compounds and synthetic analogs. Only natural occurring vitamin A active retinoids will be further discussed. A diversity of end groups and several geometrical configurations of vitamin A active retinoids are described. The major naturally occurring retinoids are compiled in fig 1.6.

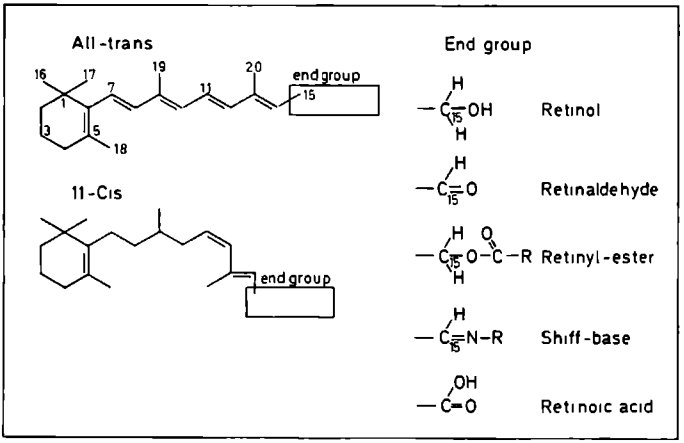


Fig. 1.6
Structure and nomenclature of the major naturally occurring retinoids.

1.4.1.2. Functions in relation to structure of the retinoids.

Vitamin A active compounds are essential to maintain a variety of normal functions: growth, differentiation and integrity of epithelial tissue, bone remodeling, reproduction and vision (cf Underwood, 1984). Some functions of the retinoids appear to be dependent on the end group and/or geometrical configuration. Generally, the all-trans configuration of the retinoids provides a higher biological activity than the cis isomers. Except in vision, where the 11-cis (retinaldehyde) configuration appears to be essential. Further, retinoic acid is able to maintain growth and differentiation equally well as retinol, but it cannot support reproduction (Thompson et al., 1964) and vision (Dowling and Wald, 1960).

1.4.1.3. Nutrition and storage.

Animals are not capable of de novo synthesis of vitamin A active substances and therefore, require external sources. The dietary source of vitamin A active retinoids is provided by carotenoids and by retinol and its esters, which are absorbed by the intestinal mucosa cells. This incorporation process requires solubilization of the retinoids in a mixed micellar solution (El-Gorab and Underwood, 1973; El-Gorab et al., 1975). The absorbed retinoids, including those which are formed in the mucosa from carotenoids, become

esterified to a saturated fatty acid (Gray et al., 1940; Eden and Sellers, 1950; Helgerud et al., 1982, 1983). Association with chylomicrons precedes the entrance to the blood circulation via the lymphatic system. The retinylesters remain associated with the chylomicron remnants which are subsequently internalized in liver cells through specific receptors (Sherill et al., 1980; Windler et al., 1980; Shelburne et al., 1980). Hydrolysis and re-esterification precedes storage of the retinoids in fat storing liver cells, which represents the major vitamin A depot in vertebrates. (Goodman et al., 1965; Lawrence et al., 1966).

1.4.2. RETINOID BINDING PROTEINS.

Retinoids represent a group of compounds which are hydrophobic and sensitive to oxidation. They require a specific micro environment, providing protection and transport facilities in aqueous environments. These requirements are met for the storage mode by the retinylester configuration and for the post-hepatic transport mode by retinol bound to a retinoid binding protein. So far a restricted number of retinoid binding proteins have been detected, most of them are not tissue specific. Table 1.1 compiles the presently known binding proteins together with some biochemical features.

1.4.2.1. Transport proteins in non-retinal tissue.

Serum Retinol Binding Protein (sRBP).

sRBP is a small protein, synthesized in the liver as a precursur of 24 kD which is rapidly processed to apo-sRBP (Soprano et al., 1981). Apo sRBP binds retinol, resulting from retinyl ester hydrolysis, and is secreted in the blood. The holoprotein forms a complex with transthyretin, which is synthesized in the liver as well. The retinol-sRBP-transthyretin complex delivers retinol through specific receptors to target tissues like intestinal mucosa, testicular cells, retinal pigment epithelium (Rask and Peterson, 1976; Heller, 1975; Chen and Heller, 1977). Subsequently, the complex dissociates into apo-sRBP and transthyretin.

Retinoid deficiency inhibits sRBP secretion and under these conditions sRBP accumulates in the liver (Muto et al., 1972; Smith et al., 1973). Repletion of vitamin A induces a rapid secretion of sRBP (Smith et al., 1973; Smith et al., 1980).

Table 1.1. RETINOID BINDING PROTEINS.

CHARACTERISTICS

BINDING PROTEINS	M_r	natural[a] ligand	Kd (M)	# binding sites	ligand[b] specificity	localization
EXTRACELLULAR						
serum retinol binding protein	21 kD	ReOH	$2x10^{-7}$	1	-	serum liver
interphotoreceptor retinol binding protein[c]	144 kD	ReOH Re=O	$1x10^{-6}$	1-2	-	eye
INTRACELLULAR						
cellular retinol binding protein	15 kD	ReOH	$15x10^{-9}$	1	+	many tissues
cellular retinoic acid binding protein	15 kD	ReCOOH	$5x10^{-9}$	1	+	many tissues
cellular 11-cis retinoid binding protein	33 kD	11-cis ReOH 11-cis Re=O	N.D.	1	+	eye
rhodopsin	39 kD	11-cis Re=O	N.D.	1	+	eye

a: ReOH symbolizes retinol, Re=O retinaldehyde and ReCOOH retinoic acid.
b: ligand specificity with respect to the C-15 moiety of the retinoid (-: not specific, +: specific). Cellular 11-cis retinoid binding protein and rhodopsin demonstrate high specificity for the 11-cis configuration as well.
c: molecular weight of bovine IRBP. Primate IRBP has a M_r of approximately 135 kD.
N.D.: not determined.

Cellular Retinol- and Retinoic Acid Binding Protein (cRBP and cRABP).

cRBP and cRABP are small retinoid binding proteins with similar physico-chemical characteristics. They are present in several tissues in a variety of species (cf Chytil and Ong, 1984). These retinoid binding proteins probably serve as receptors for retinoid from the blood circulation and as transport vehicles to cellular sites. Striking correlation were reported between the presence of the binding proteins and the responsiveness of a cell to retinoids (Schindler et al., 1981; Sherman et al., 1981). Contradictory results were obtained as well (Breitman et al., 1981; Douer and Koeffler, 1982). In its effect on cell differentiation, vitamin A probably interacts with nuclear components, binding sites for cRBP were detected in the nucleus (Takase et al., 1979; Liau et al., 1981). The role of the cellular binding proteins (cRBP and cRABP) in mediating the various physiological effects of vitamin A is not yet understood and subject to intensive research.

1.4.2.2. Transport proteins in retinal tissue.

The retinoid binding proteins relevant to the visual cycle will be considered in more detail. Allthough cellular retinoic acid binding protein is present in the retina, it is not functional in the visual cycle, since its ligand, retinoic acid, is only irreversibly formed from retinaldehyde (Futterman, 1962) and therefore, is not a precursor of the chromophoric group of rhodopsin. Table 1.2. compiles some additional properties of the retinoid binding proteins in the retina.

Cellular Retinol Binding Protein (cRBP).

In analogy to the discussed function of cRBP in non-retinal tissue, transfer of the retinoids from the plasmalemma of the RPE to intracellular sites requires a transport vehicle through the cytoplasma. Allthough solid evidence is lacking, the common notion is that cRBP serves to transport retinol between apical and basal plasma membrane and sites of esterification. Some support is provided by the faster esterification of retinol bound to cRBP than free retinol (Berman et al., 1980). Whether cRBP transport is required for retinol dependent eptithelial differentiation of the RPE and retina is not known.

Table 1.2. RETINOID BINDING PROTEINS IN RETINAL TISSUE.

	AMOUNT (pmol/bovine eye)			ENDOGENOUS LIGANDS		
BINDING PROTEIN	RPE	RETINA[a]	IPM	RPE	RETINA	IPM
cRBP	600	100	—	at ReOH	at ReOH	
cRAlBP	700	1000	—	11-c Re=O	11-c Re=O (75%) 11-c ReOH (25%)	
IRBP[b]			3000			at ReOH 11-c ReOH 11-c Re=O at Re=O
RHODOPSIN	*	50,000	--		11-c Re=O	

cRBP is the abbreviation of Cellular retinol binding protein, cRAlBP of cellular 11-cis retinoid binding and IRBP of interphotoreceptor retinoid binding protein. ReOH refers to retinol, Re=O to retinaldehyde, IPM to interphotoreceptor matrix, 11-c to 11-cis retinoids and at to all-trans.
a: cRBP is localized in extensions of the Muller cells in the plexiform layer, cRAlBP is detected throughout the Muller cells with heavy labeling at the external membrane.
b: IRBP retinoid burden is light dependent. In light adapted eyes, all-trans retinol presents the major burden, in dark adapted eyes 11-cis retinaldehyde.
*: phagocytized opsin.

Cellular 11-cis Retinoid Binding Protein (cRAlBP).

cRAlBP is exclusively located in retinal tissue and provides protection of the 11-cis retinoids to modifying agents (Saari and Bredberg, 1982). Besides opsin, this is the only retinoid binding protein which specifically binds 11-cis retinoids (retinol and retinaldehyde). Therefore, it appeared attractive to ascribe cRAlBP a major role in the visual cycle. The rate of transition of 11-cis retinol or 11-cis retinaldehyde appears to be enhanced upon binding to cRAlBP (cf section 6.1). However, its putative role in regeneration of rhodopsin (combining 11-cis retinaldehyde with apo protein opsin) has to be approached critically. The affinity of cRAlBP for 11-cis retinaldehyde is higher than the affinity of opsin or IRBP (see below) (Saari and Bredberg, 1986). Therefore this transfer is not likely to occur.

Interphotoreceptor Retinoid Binding Protein (IRBP).

The interphotoreceptor retinoid binding protein is a large glycoprotein which proved to be able to bind besides retinoids, fatty acids and a-tocopherol (Fong et al., 1984). This extracellular protein might be considered 'the serum albumin of the retina'. Due to its localization, IRBP is thought to fullfil a shuttle function in the visual cycle, carrying retinoids between the outer segments and the other cells bordering the interphotoreceptor matrix. Specific receptors for IRBP are probably not involved in delivery of retinol through specific receptors for IRBP since these are not present e.g. on the plasmalemma of the RPE cells (Hollyfield et al., 1985b).

1.4.2.3. Non-transport proteins.

Rhodopsin.

Unlike transport proteins, rhodopsin binds retinoids covalently through a Schiff-base. This membrane glycoprotein functions as a complex with its prosthetic group, 11-cis retinaldehyde, in photoreception and has no transport function. Light capture causes isomerization of 11-cis retinaldehyde and initializes a transient reaction sequence, which finally results in the sensation of sight.

1.4.3. THE VISUAL CYCLE.

The pathway traversed by vitamin A from bleaching all the way to regeneration of rhodopsin is termed the visual cycle. The general aspects will be reviewed briefly and are summarized in fig 1.7. The participation of the RPE cells in the visual cycle will be considered in more detail in chapter 5 and 6.

1.4.3.1. The steps following bleaching.

Photo-activation of rhodopsin involves photo-isomerization of the 11-cis retinaldehyde merely to the all-trans isomer (Wald, 1968). This triggers conformational changes in the protein, which activate the transduction system. The all-trans retinaldehyde eventually leaves the protein (photolysis), where upon it is reduced by a membrane bound retinaldehyde oxidoreductase (cofactor NADPH) to all-trans retinol (Futterman, 1963; de Pont et al., 1970; Bridges, 1977). The all-trans retinol then leaves the outer segment, traverses the inter photoreceptor matrix to the retinal pigment epithelium cells. The all-trans retinol enters through the apical membrane and is stored as a retinyl-fatty acid ester (Dowling, 1960; Zimmerman, 1974; Bridges, 1976). The general outline of this branch of the visual cycle is well documented, but the discrete steps are not yet very well characterized. Nevertheless, this part of the visual cycle is still better characterized than the reverse branch: effectuation the

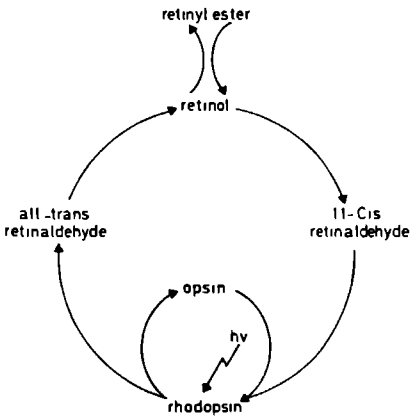


Fig. 1.7
Overall conversions of retinoid in the visual cycle.

regeneration of rhodopsin by incorporation of 11-cis retinaldehyde.

1.4.3.2. The regeneration process.

The regeneration process is a sequence of retinoid transitions resulting in the conversion of retinylester into the chromophoric group, including retinoid transport and binding by opsin to form rhodopsin. In the light, the retinal tissues continuously have to cope the necessity to regenerate bleached rhodopsin. In this process the following subdivision can be made:

1. Conversion of the retinylester into 11-cis retinaldehyde which comprises hydrolysis, isomerization and oxidation. (The biochemistry of the retinoid modifications will be surveyed in detail in chapter 6).

2. Transport of retinoids to the outer segments.

The current model of the visual cycle (Bridges et al., 1984) is displayed in fig. 1.8. The major questions of the visual cycle are where and which retinoid is to be isomerized (all-trans to 11-cis) and oxidized (retinol to retinaldehyde). The model postulates that isomerization to 11-cis takes place at the alcohol level in the

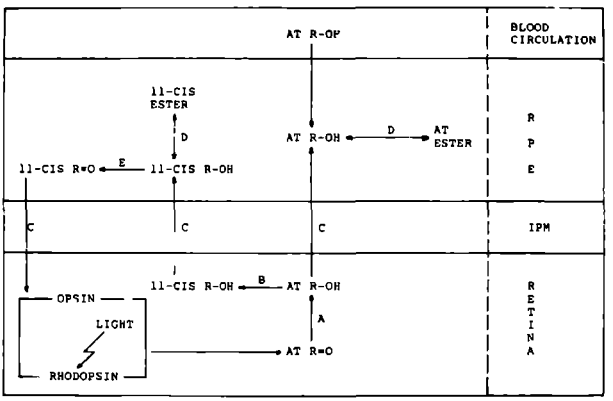


Fig. 1.8
Model for the visual cycle in the mammalian eye.
A: all-trans retinol oxidoreductase; B: isomerase; C: transport through the interphotoreceptor matrix; D: esterase; E: 11-cis retinol oxidoreductase. The following abbreviations are used. AT: all-trans; R-OH: retinol; R=O: retinaldehyde; ESTER: retinylester; IPM: interphotoreceptor matrix.

Müller cells. Oxidation to 11-cis retinaldehyde takes place in the retinal pigment epithelium cells. Inter cellular transport would be carried out by interphotoreceptor retinoid binding protein (IRBP).

This model of the visual cycle, manifesting a rather complex construction, essentially is based on the following observations:

Retinoid transitions.

i. the inability of the isolated retina to regenerate visual pigment* (unless provided with 11-cis retinaldehyde), ii. the unambiguous requirement of the presence of the RPE cells (see chapter 5), iii. complete mystery where the crucial retinoid conversion the isomerization, is located, iv. the presence of a stereospecific 11-cis retinol oxidoreductase in the RPE cells and v. the distribution of cRAlBP and its endogenous ligand (table 2). For further details see chapter 5 and 6.

Retinoid transport.

Upon bleaching, the all-trans retinol load increases about 5 times (to $\pm$ 15% saturation of the binding sites). During dark adaptation, its load of 11-cis retinaldehyde increases to 5 - 10% saturation. Although, solid evidence for its proposed transport function is lacking (e.g. no receptor sites are found at the apical plasmalemma of RPE), the dependence of the retinol load on the light history suggests a role in chromophore transfer in both branches of the visual cycle.

*Footnote: The isolated amphibian retina is able to regenerate the visual pigment with 11-cis retinol since a 11-cis retinol oxidoreductase is present, which is lacking in the mammalian retina.

1.5. ROD OUTER SEGMENT RENEWAL AND PHAGOCYTOSIS.

The pioneering work of R.W. Young established that the disc membranes of rod photoreceptor outer segments (ROS) are constantly being renewed throughout life (Young, 1967). The assembly of new discs, at the basis of the outer segment, is counteracted by a process of phagocytosis of their tips (Young and Bok, 1969) and degradation within the retinal pigment epithelium cells (Young and Bok, 1969; Ishikawa and Yamada, 1970). The direct correlation between rod outer segment turnover and opsin turnover has been established by Hall et al. (1969).

1.5.1. ROD OUTER SEGMENT RENEWAL.

The continuous renewal of discs at the basis of the outer segments implies a continuous supply of their components. Disc membrane components, synthesized in the inner segment of the photoreceptor cell, are exported to the outer segment by a transport mechanism described by Papermaster and coworkers (Papermaster et al., 1975; Papermaster and Schneider, 1982; Papermaster et al., 1985). The major components of the rod outer segments are rhodopsin (60% on total outer segment protein base) and the phopholipids (60 phospholipids/rhodopsin).

1.5.1.1. (Rhod)opsin biosynthesis.

Autoradiographic studies, with labeled amino acids, demonstrated opsin synthesis at the rough endoplasmic reticulum of the photoreceptor inner segment. Opsin 'in statu nascendi' traverses the Golgi apparatus (glycosylation is initiated in the rough endoplasmic reticulum and completed in the Golgi apparatus), accumulates in the apex of the inner segment and is subsequently incorporated into graving outer segment discs (cf Young 1976; Bok, 1985). Incorporation of the chromophoric group occurs in the outer segments (Defoe and Bok, 1983).

In animals reared in cyclic light, no light effects are observed on the rate of opsin biosynthesis (Dudley et al., 1984). However, prolonged dark or light conditions, respectively reduces or elevates this rate (Hollyfield and Anderson, 1982).

1.5.1.2. Lipid biosynthesis.

Phospholipids (the major lipid in ROS) demonstrate, as compared to opsin, a much more dynamic behavior. Newly synthesized phospholipids become randomly dispersed throughout the ROS (Bibb and Young, 1974), and show a faster turnover than opsin (18-23 vs 40-50 days in frog) (Anderson et al., 1980a-d).

The lipid matrix of the outer segment disc membranes is highly unsaturated (over 50% polyunsaturated acyl chains), which appears to be essential for proper functioning (Wheeler et al., 1975). Dietary deprivation of essential fatty acids, which are precursors for these unsaturated lipid-acyl chains, leads to rapid depletion of most tissues except for retina (and brain) which tenaciously holds onto its level of unsaturation (Anderson and Maude, 1972). In view of the high turnover of the outer segments, recycling mechanisms probably exist, which conserve the precious, highly unsaturated fatty acids for the outer segment. The physiological function of the high degree of unsaturation remains to be elucidated.

Unlike opsin biosynthesis, phospholipid synthesis displays a circadian rhythm (Dudley et al., 1984). Animals reared in cyclic light, show, during the light period, an elevated rate of synthesis of the major phospholipids: phosphatidyl-ethanolamine, -choline and -inositol (radioactivity incorporationin phosphatidylserine was to low to be detected.

1.5.2. CIRCADIAN RHYTHM

The steady state length of the outer segment, despite the ongoing renewal at its base, implies a balanced removal of discs at its apex which has been called: disc shedding. The phagocytotic role of the RPE cells in this process was first demonstrated by Young and Bok (1969). Currently, disc shedding includes phagocytosis of the disc package by the RPE cells

In 1976 LaVail described the (circadian) rhythmic nature of the disc shedding. In animals reared in cyclic light, the majority of the ROS discs detached shortly after the beginning of the light period (fig 1.9). In many species, shedding of cone outer segment discs occurs after beginning of the dark period, although deviations are observed in some species.

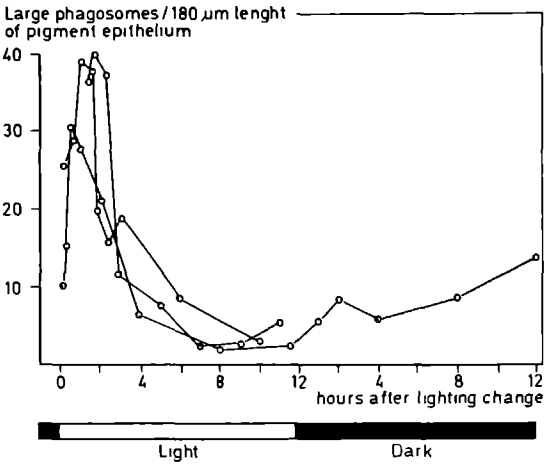


Fig. 1.9
Outburst of phagocytosis shortly after begirning of light period.
Explosive increase of number of phagosomes in the (rat) RPE shortly after light onset and rapid subsequent degradation of phagosomes. This process follows a circadian rhythm.

The phenomenon of rhythmic shedding in rods appears to be a feature of all vertebrates examined thus far. Below, unless stated otherwise, the disc shedding in rat will be discussed.

1.5.2.1. Role of light.

Light acts as a 'zeitgeber' for synchronizing the shedding rhythm with the external light cycle (described for rat, nevertheless this is probably a common feature in homoiothermic animals). A burst of rod shedding occurs soon after light onset and the rhythm persists in constant darkness, with the shedding peak attenuated and extended (LaVail, 1976, 1980; Goldman et al., 1980). The phase of the rhythm can be shifted (phase delay occurs slowly: ca 4 weeks; phase advance occurs in several days, (LaVail, 1980; Goldman et al., 1980)). The burst of rod shedding however does not occur in constant light, a dark period of at least 2 hr is necessary to induce a shedding event in the subsequent light period. When animals switched from constant light to constant darkness, the rhythmic shedding process is resumed corresponding to the originally entrained light/dark cycle. For this reason, it has been argued that inhibition in constant light is due to a local

effect on some chemical process, rather than to deregulation of the circadian oscillator. This view is supported by the observation that this light inhibition of shedding manifest itself only in the exposed eye (see fig 1.10) and further, by the difference in light sensitivity to accomplish phase advance or inhibition of shedding (Tierstein et al., 1980; Baker et al. 1986b).

1.5.2.2. The circadian oscillator.

Several observations suggest that a circadian oscillator exists within each eye. Humoral factors do not seem to be essential, since the circadian rhythm persists after removal of the pineal gland, the superior cervical ganglia, the hypophysis and the thyroid-parathyroid glands (Tamai et al., 19878; LaVail and Ward, 1978). This conclusion is consistent with the observation on monocularly occluded rats (fig 1.10). Further, the shedding rhythm persists after transection of the optic nerve (Goldman et al., 1980; Tierstein et al., 1980) and in excised Xenopus laevis eyes (Flannery and Fisher, 1979, 1984). The disability to entrain disc

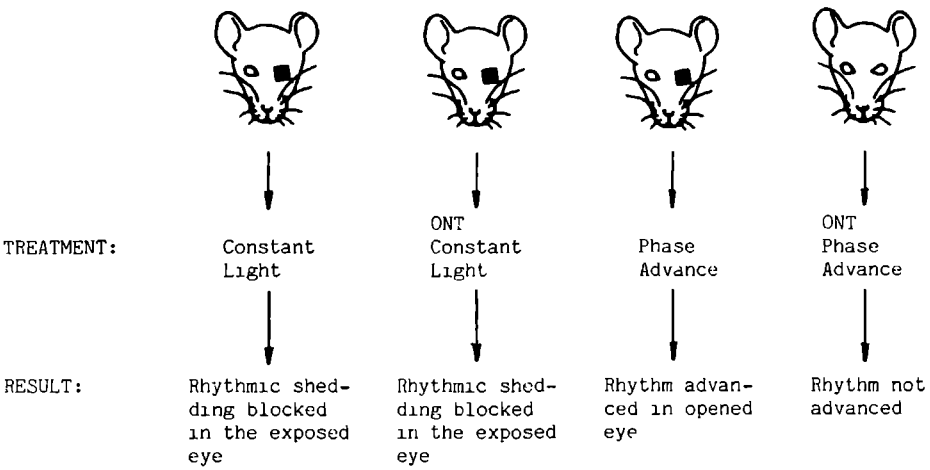


Fig. 1.10
Control of disc-shedding occurs within the eye.
Eye patch experiments. The basic control of disc-shedding and subsequent phagocytosis are intrinsic features of the eye. ONT refers to optic nerve transection, phase advance to a shift of the beginning of the light period to an earlier time.

shedding to a new light cycle, after transection of the optic nerve, inferred that a circadian oscillator exists in each eye, albeit cued to the environmental light cycle by signals from a synchronizer in the central nervous system.

In the leopard frog (Rana pipiens), the rhythm of disc shedding appears to be driven largely by the dark/light transition, since both constant darkness and constant light completely block the shedding (Hollyfield et al., 1976; Basinger et al., 1976). The shedding rhythm in the African claw frog (Xenopus Laevis) has features common to both rat and R. pipiens (Besharse et al., 1977, 1980; Besharse and Dunis, 1982).

1.5.2.3. Intraocular factors controlling disc shedding.

Enzymes involved in the synthesis of pineal rhythm generators are also present in the eye. These enzymes, N-acetyl transferase and hydroxyindole-O-methyltransferase, undergo diurnal fluctuations of activity in the retina (Nagle et al., 1972; Binkley et al., 1979; Hamm and Menaker, 1980) as do their respective products, N-acetyl serotonin (Pang et al., 1981) and melatonin (Pang et al., 1980). The development of an in vitro system, X. laevis eyecup preparation, gave great impetus to research on the role of the indoleamines. Administered melatonin produced disc shedding in eyecups of animals maintained in constant light for 4 days (Besharse and Dunis, 1983). Besharse and Iuvone (1983, Iuvone and Besharse, 1983) were able to demonstrate circadian rhythmic activity of N-acetyl transferase in the eyecups and could induce a phase shift. Although the evidence in favor of the methoxyindoles as regulating factor in the circadian disc shedding events accumulates, we are far from understanding the exact regulatory mechanism underlying disc shedding. The X. laevis eyecup experiments reveal a diversity of disc shedding affectors. In addition to the indoleamines several compounds (high K^+, low Na^+, low divalent ions, bicarbonate, ouabain, several (excitatory and non-excitatory) amino acids) exhibited strong effects on the shedding event (Greenberger and Besharse, 1983, 1985; Williams et al., 1984). Unlike melatonin, these compounds evoke light-independent shedding events. Their physiological relevance, if any, remains to be established.

1.5.3. MECHANISMS OF DISC PACKAGE DETACHMENT.

Disc shedding is usually described as a process of detachment of packages of discs, from the photoreceptor distal tip, with subsequently phagocytosis by the RPE. A major question is whether one or two processes are involved i.e. do the photoreceptors shed independently or does the RPE 'bite off' pieces of the outer segment (Spitznas and Hogan, 1970; Besharse and Dunis, 1982; Young, 1971; Currie et al., 1978).

Recently, some relevant observations were described. In 1985, Matsumoto and Besharse observed in X. laevis, that at the point of incipient disc separation outer segments stain with fluorescent dyes, which normally cannot permeate plasma membranes. This effect could not be induced in retinas isolated prior to light onset. Secondly, activation of shedding leads to the formation of RPE pseudopods, which envelope ROS-tips, prior to disc detachment (Besharse et al., 1984). These observations implicate the RPE cells as an active partner in the disc detachment process. Cytochalasin inhibits the pseudopod formation and... disc detachment! Nevertheless, vesiculation of the rod outer segment at the incipient separation point was still observed, suggesting that outer segments are actively involved in the process as well.

1.5.4. PHAGOCYTOSIS OF ROD OUTER SEGMENT DISC PACKAGES.

Whatever the mechanism of detachment, phagocytosis, the actual incorporation in the RPE cells of the (shedded) disc packages, follows rapidly. Phagocytosis can be divided into three phases: 1. recognition and attachment, 2. internalization and 3. degradation.

1.5.4.1. Recognition and attachment.

The mechanism of recognition is still not understood. Putative mediators are carbohydrate residues on the surface of the outer segment plasma membrane, which might serve as recognition sites for receptors (which are putative as well) on the apical surface of the RPE (O'Brien, 1976). Some support was obtained by the observation that carbohydrates, present in the outer segment glycocalyx, inhibit phagocytosis (Heath and Basinger, 1983). In this model, a light dependent alteration of the oligosaccharide of opsin,

embedded in the outer segment plasmamembrane, was considered to be involved in shedding and phagocytosis. However, no differential lectin binding to the apical part of the outer segments dependent on illumination conditions could be detected (Bridges and Fong, 1979; McClaughin and Wood, 1980). Inhibition experiments of the binding of opsin-liposomes to RPE cells with a variety of carbohydrates (Kean et al., 1986) also do not provide any positive indication that carbohydrates are involved in recognition or attachment.

Evidence for a specific receptor for ROS-disc packages on the apical RPE surface is lacking, allthough several observations suggest a certain degree of specificity (Hollyfield and Ward, 1974; Hollyfield, 1976). In RPE cell cultures, the substrate-preference for endocytotic activity decreases in the order: ROS - yeast, polystyrene - bacteria - red blood cells (Hollyfield, 1976; Mayerson and Hall, 1986).

1.5.4.2. Internalization.

Whatever the precise mechanism for recognition and attachment, internalization of the incipient phagosome occurs quickly and is apparently triggered by an as yet poorly defined transmembrane signaling process. In the RPE cells, the formation of subplasmalemmal actin plaques ('feltwork') is induced at the ROS attachment site (Chaitin and Hall, 1983b). The actin feltwork is not formed in the RPE of the RCS-rat, which is defective in phagocytosis of outer segments, but ingests artificial substrates equally well as normal RPE cells (Edwards and Flaherty, 1986). Uptake of disc packages is inhibited by cytochalasin (Besharse and Dunis, 1982).

Once internalized, the phagosomes are transported rapidly to the basal RPE cytoplasm while they fuse with the lysosomes and are degraded (Ishikawa and Yamada, 1970; Spitznas and Hogan, 1970; Herman and Steinberger, 1982a,b). Cytoplasmic microtubules have been implicated in the transport (Burnside, 1976). Treatment with colchicine indeed inhibits cytoplasmic transport and impedes degradation (Besharse and Dunis, 1982; Herman and Steinberg, 1982a,b).

1.5.4.3. Degradation.

High activities of several lysosomal enzymes have been described in RPE cells (proteases of the cathepsin D type, catalase, acid phosphatase, phospholipase A1 + A2, α-fucosidase, α-mannosidase, N-acetyl-β-glucosaminidase, β-galactosidase) (Hayasaka et al., 1975; Berman, 1979; Regan et al., 1980; Woo-Kuen et al., 1981; Hayasaka and Shiono, 1982; Zimmerman et al., 1983; Seyfried-Williams and McClaughlin, 1984; Tsung et al., 1984). The specific activities of the hydrolytic enzymes are even higher in RPE cells than in liver. This indicates a large digestive potential of these cells.

Rapidly following endocytosis, the degradation of the phagosome is initiated, a process which has been described in detail morphologically (Young and Bok, 1969; Bok and Young, 1979; Herman and Steinberg, 1982a,b). First, the plasma membrane surrounding the disc package is rapidly digested and subsequently the membraneous structure gradually disappears with progressing degradation (cf Bok and Young, 1979).

The phagocytotic processing of opsin, phospholipids, poly-unsaturated fatty acids and the chromophoric group will be considered briefly.

Opsin.

The RPE cells every day have to cope with the internalization and degradation of a large amount of ROS material. Bovine RPE (5 x 10^6 RPE cells/eye) phagocytizes daily ± 10% of the total opsin (50 nmol/eye). This phagocytotic burden represents approximately 2000 discs/RPE cell·day. In rat, one RPE cell even has to deal with ± 7500 discs/day (Bok and Young, 1979). Evidence has been presented for phagosomal degradation into small peptides or amino acids, which probably enter the regular RPE pool and are partially recycled to the rod cell. The degradation of opsin will be further discussed in chapter 7.

Phospholipids.

The phagocytosis of 1000 pmoles opsin/10^6 RPE cells · day, yields 60,000 pmoles of phospholipids/10^6 RPE cells · day to be processed. This large amount of highly unsaturated ROS phospholipids amounts to approximately three times the endogenous phospholipid pool (ca 20 nmol/10^6 RPE cells, Berman et al., 1974).

The lipids are probably processed into their building blocks.

Poly-unsaturated fatty acids.

The retina is able to maintain its high degree of unsaturation during dietary deprivation of essential fatty acids (Anderson and Maude, 1972). Hence, oxidative degradation is unlikely; probably the fatty acids are recycled to the rod cell. Little is known about fatty acid metabolism in the phagosomes. Processing (and subsequent transport to the retina) is presumably carried out rapidly, since hardly any increase of highly unsaturated fatty acids could be detected during the phagocytotic process (Baker et al., 1986a).

Chromophoric group.

The retina hold its retinoid pool very efficiently against dietary vitamin A deprivation. The rhodopsin content in vitamin A deficient rats, reared in the dark, remains constant for several months (Noell et al., 1971). Assuming, no recycling of the phagocytized chromophoric group occurs, rhodopsin should be lost in 10 - 20 days (even in the dark). Hence, it is not likely that the 11-cis retinaldehyde released during degradation of rhodopsin undergoes catabolic degradation. Probably, the retinaldehyde is reduced and stored as retinylester.

1.6. AIMS AND OUTLINE OF THIS INVESTIGATION.

The preceding introduction demonstrates the essential role of the retinal pigment epithelium in supporting the physiology of the retina and photoreceptor in particular. In order to investigate the properties of the RPE cell in more detail, the intact eye or even the isolated eyecup is not very suitable, because of the presence of a large variety of cell types in addition to RPE. Yet, an obvious instrument for studies on RPE, isolated RPE, have not been used convincingly, since this requires isolation of intact RPE cells (a monolayer of fragile cells surrounded by a jungle of other cells) in high yield. For that purpose, the most popular methods used either brushing (Glocklin and Potts, 1962, further adapted by Berman and Feeney, 1976) or enzymatic isolation. The former was shown to yield seriously damaged cells (Saari et al., 1977), the latter will affect plasma membrane proteins and potentially disturb the communication with the environment. These considerations tempted Bridges (1984) to state: "it is exceedingly difficult to

prepare RPE cells with intact plasma membranes". Hence, an appropriate isolation procedure for RPE cells was urgently needed.

The aim of this investigation was to develop an isolation procedure for bovine retinal pigment epithelium cells which would produce a high yield of intact cells. The isolated cells should provide a suitable substrate for further investigation of the interrelation between RPE and photoreceptor cell (vitamin A metabolism, phagocytosis), either in a long term tissue culture or a short-term incubation system.

In collaboration with Prof. Dr. Edward A. Dratz (on sabbatical leave in our laboratory in 1982) and Dr. J. Thijssen (Dept. of Ophthalmology, CER Nijmegen), we succeeded in developing a procedure to isolate intact RPE cells. This procedure is based on perfusion of the isolated bovine eye with an isotonic salt solution free of divalent cations (chapter 3). Although, we were able to culture the isolated bovine RPE cells, unfortunately, it appeared that in culture the bovine RPE cells rapidly dedifferentiate and cannot provide reliable information on the properties of RPE cells in vivo (chapter 4). Therefore, chapter 5 describes an alternative approach: the development of a short-term incubation system in which RPE cells remained viable. With the energy charge and retention of small cytosolic components as parameters we could demonstrate that RPE cells in short-term incubation fulfulled our requirements.

This system was then utilized to investigate two important functions of RPE cells: retinoid metabolism (chapter 6) and phagocytosis (chapter 7).

Chapter 2

METHODS AND TECHNIQUES.

This chapter compiles methods and techniques which are used in the studies described in several of the following chapters: retinoid analysis, determination of cell density and electro-immunoblotting. Chapter-specific techniques will be described in the particular chapter.

2.1. RETINOID ANALYSIS.

In the visual cycle, geometric isomers of various retinoids play an essential role. Both the 11-cis and all-trans conformations of retinol, retinaldehyde and retinylester have been shown to occur in the visual cycle (Wald, 1968). Studies on retinoid metabolism in relation to the visual cycle require analytical techniques, which permit quantitative determination of this complex group of compounds. Furthermore, special care should be taken in handling these hydrophobic and relatively labile compounds, which are susceptible to chemical and geometrical modification by a variety of agents (cf Groenendijk et al., 1980a,b).

In view of these considerations we looked for a sensitive technique allowing quantitative analysis of all retinoids involved in the visual cycle. Separation of retinoids by means of HPLC as described by Groenendijk et al. (1980a) appears to have a large potential as a fast and non-destructive procedure. This technique was therefore optimized for analysis of retinoids of retinal pigment epithelium cells.

2.1.1. MATERIALS.

All-trans retinaldehyde and all-trans retinylpalmitate were obtained from Kodak Co (Rochester, New York, USA). Hexane (HPLC-grade) was obtained from Fisons (Loughborough, England), sodium borohydride and Ammonyx LO from Fluka AG (Buchs, Switzerland) and dichloromethane, methanol, triethylamine, dioxane and iso-octane (LiChrosolv-grade) from E. Merck (Darmstadt, W-Germany). LiChrosorb Si 60-7 cartridge columns (100 x 3 mm) and Silica guard columns (10 x 3 mm) were obtained from Chrompack (Middelburg, The Netherlands). All reagents were at least of analytical grade.

2.1.2. SEPARATION AND QUANTIFICATION OF RETINOIDS BY HPLC.

2.1.2.1. Separation of retinoids by isocratic HPLC.

A retinoid standard mixture was prepared as described before (Groenendijk et al., 1980a): all-trans retinaldehyde in ethanol was photoisomerized (50°C; >435 nm; 2-3 hr under nitrogen) to give a mixture of mainly all-trans, 13-cis and 11-cis retinaldehyde. A part of this mixture was reduced with $NaBH_4$ to the corresponding alcohols. After extraction with hexane the retinoid solution was stored under an inert atmosphere (Argon) at -70°C. Aliquots of the isomeric mixture of retinaldehyde and retinols were mixed with retinyl-palmitate (Eastman Kodak Co, Rochester, New York, USA) and syn all-trans retinaloxime (prepared as described by Groenendijk et al., 1980a) to produce the retinoid standard mixture (0.5 to 1.5 nmoles per component/ml).

Satisfactory separation was obtained by straight-phase HPLC (Varian 8500 isocratic syringe pump with a Valco injector, which was equipped with a LiChrosorb Si 60-7 column (100 x 3 mm) and a silica guard column (10 x 3 mm) using hexane : dioxane (95:5) as an eluent at a flow rate of 0.5 ml/min and detection (Schoeffel SF 770 detector) at 328 nm and 0.01 AUFS (fig. 2.1). Detection limit: 0.2 pmol/HPLC run.

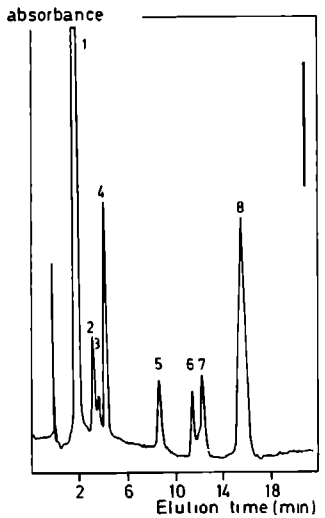


Fig. 2.1.
Isocratic HPLC elution profile of retinoid standard mixture.

Elution profile of a standard mixture of retinoids attained by straight phase HPLC in the isocratic mode. Column: LiChrosorb Si 60-5 (100 x 3 mm); eluent: 95% hexane/ 5% dioxane; flow rate: 0.5 ml/min; detection: 328 nm. Bar represents 0.0025 absorbance units. Peak 1: retinyl-palmitate; peak 2: 11 + 13-cis retinal-dehyde: peak 3: 9-cis retinaldehyde; peak 4: all-trans retinaldehyde; peak 5: syn all-trans retinaloxime; peak 6: 11-cis retirol; peak 7: 13-cis retinol; peak 8: all-trans retinol.

2.1.2.2. Quantification of retinoids.

In order to compensate for experimental variations, quantitative determination requires the use of an internal standard. Such an internal standard (i) should have physico-chemical characteristics comparable to the compounds of interest, (ii) should not interfere with their HPLC separation and (iii) should not occur in the tissue under investigation. Syn all-trans retinaloxime, which is a derivative of retinaldehyde, appeared to be an attractive candidate (Groenendijk et al., 1980a), since its hydrophobicity is of the same order as the other retinoids and its λ max (357 nm in hexane) allows detection at 328 nm. Syn all-trans retinaloxime is a non-natural retinoid and not present in ocular tissues. In our HPLC system, this compound elutes 5 min later than all-trans retinaldehyde and 3 min before 11-cis retinol and does not interfere with the separation of the ocular retinoids (fig 2.1).

The reliability of syn all-trans retinaloxime as an internal standard for peak-area integration was further investigated by carrying out several HPLC separations (n=6) over a period of two weeks, thereby injecting 15 µl of standard retinoid mixture per run. Table 2.1 presents average retention times and quantitative calculations on the basis of syn all-trans retinaloxime as internal standard. Data reduction was performed on the integrated areas, thereby correcting for differences in molar extinction coefficient (Groenendijk et al., 1980a) by a Hewlett Packard 3353 A integrating system which converted peak areas into nanomoles per original sample volume. Satisfactory results were obtained with maximal deviations of about 5%. These results were not significantly affected by using variable injection volumes (5 to 50 µl) (not shown).

This demonstrates that syn all-trans retinaloxime behaves as a suitable internal standard for the quantitative analysis of retinoids by HPLC.

2.1.3. OPTIMIZED EXTRACTION PROCEDURE OF RETINOIDS FROM RETINAL PIGMENT EPITHELIUM.

Investigation of the vitamin A metabolism in RPE cells requires, next to reliable separation and quantification, reproducible extraction of the entire retinoid population (all isomeric

Table 2.1 RELIABILITY OF SYN ALL-TRANS RETINALOXIME AS AN INTERNAL STANDARD FOR QUANTITATION OF OCULAR RETINOIDS VIA HPLC-SEPARATION

RETINOID	QUANTITY[a]	RETENTION TIME[b]
retinylester	189 ± 4%	1.82 ± 0.5%
retinaldehyde		
11-cis (+13-cis)	21 ± 3%	3.30 ± 0.7%
9-cis	6 ± 5%	3.85 ± 0.9%
all-trans	37 ± 4%	4.32 ± 0.9%
syn all-trans retinaloxime	159	9.01 ± 1.1%
retinol		
11-cis	6 ± 4%	12.00 ± 2.0%
13-cis	7 ± 3%	12.98 ± 1.1%
all-trans	29 ± 4%	16.34 ± 2.0%

a: quantity: mean ± S.E % (pmol retinoid per injection) calculated using syn all-trans retinaloxime as an internal standard. Over a period of two weeks six analysis were run whereby 15 µl of retinoid standard mixture was injected 6 times.

b: retention time in minutes (mean ± S.E%)

congeners of retinol, retinaldehyde and retinylester). Extraction with dichloromethane/methanol/water (1:1:1) will result in quantitative recovery of retinol and retinylester with little risk of isomerization (Groenendijk et al., 1980a). The recovery of protein bound retinaldehyde is low, however (Groenendijk et al., 1980a). Nevertheless, the applicability of this procedure for extraction of retinoids from RPE was explored since reliable alternatives are not available.

All manipulations were carried out under dim red light to prevent photoisomerization. Extraction of retinoids from RPE was carried out at room temperature in 10-ml stoppered glass tubes. To 0.5 ml freshly isolated or incubated cells (routinely 1 - 2 x 10^6 RPE cells/tube) 6% aqueous Ammonyx LO was added to a final concentration of 0.06%. Supplementation with Ammonyx LO reduces adherence of retinoids to 'whatever surface present' and in particular enhances the recovery of 11-cis retinol (cf appendix). Even after more than 100 HPLC runs we did not observe any effect of

this amount of detergent on the column performance.

Following addition of internal standard (ca 0.5 nanomoles of syn all-trans retinaloxime in 100 µl iso-octane the extraction was initiated by adding 1.5 ml methanol and 1.5 ml dichloromethane. After every addition, the tube content was vigorously mixed (15 - 20 sec on a Vortex mixer). At this stage a single phase should have been obtained otherwise another 200 µl of methanol was added. Finally, 1.0 ml water was added to induce phase separation and after 20 sec of vigorous mixing, the organic and aqueous phase were completely separated by centrifugation (10 min; 5000 x g). The lower organic phase was collected and the upper water-methanol layer re-extracted with 1.5 ml dichloromethane.

The dichloromethane layers were pooled and 100 µl of the volatile organic base triethylamine was added. This addition appeared to be essential in reducing the relative large variation in recovery of the retinaldehydes, which originally encountered ca 20%. Probably, a slightly alkaline environment prohibits the Schiff-formation of stable protonated bases of retinaldehyde with any aminocompounds present like phopholipids.

Subsequently, the organic solvent was evaporated by a stream of nitrogen. The residue was gradually dissolved in 5% dioxane/95% hexane, by first adding 10 µl of dioxane followed by 10 µl hexane and finally another 180 µl of hexane, with vigorous mixing after every step. The samples were either directly analyzed by HPLC or stored at -20^{o}C under Argon for maximally two weeks.

The reproducibility of the total extraction and quantification procedure was checked by analyzing a dilution series of RPE cells (0.3 to 5 x 10^6 cells per extraction tube) to which a fixed amount of syn all-trans retinaloxime was added prior to extraction. The recovery of syn all trans retinaloxime was a constant 98 $\pm$ 3%. Table 2.2 shows that the retinoid content calculated from the various cell dilutions is not significantly different and that the standard error is generally not markedly larger than that of the analytical procedure itself (table 2.1). These findings exemplify the broad working range of this extraction procedure. Routinely extractions for retinoid analysis were performed on 1 - 2 x 10^6 RPE cells.

Table 2.2 RETINOID CONTENT OF RETINAL PIGMENT EPITHELIUM CELLS[a]

number of RPE cells per extraction tube	retinylester	retinaldehyde		retinol	
		11-cis[b]	all-trans	11-cis	all-trans
5.3×10^6	1603	93	21	52	47
2.6×10^6	1270	77	28	38	32
1.3×10^6	1862	103	49	52	53
0.7×10^6	1794	100	42	47	38
0.3×10^6	1473	83	50	67	38
mean ± S.E.	1600±110	91±5	38±5	51±5	42±4

a: pmol retinoid/10^6 RPE cells.

b: No reduction of the retinaldehyde fraction was carried out to determine the isomeric status. The 11-cis fraction therefore probably contains 13-cis as well.

2.1.4. ANALYSIS OF GEOMETRIC CONFIGURATION OF RETINYLESTER AND RETINALDEHYDE FRACTION.

The HPLC procedure described above does not sufficiently resolve the geometric isomers of retinylester and retinaldehyde and therefore does not allow the determination of their isomeric composition. Since base-line separation of 11-cis, 13-cis and all-trans retinol can be achieved, the isomeric composition of the retinaldehyde and retinylester fraction was determined by HPLC after their conversion by either saponification (retinylester) or reduction (retinaldehyde) into the corresponding retinols.

2.1.4.1. Isomeric composition of the retinylester fraction.

The retinylester fraction was separated from the other retinoids by semi-preparative straight-phase HPLC (LiChrosorb Si 60-5; 250 x 9 mm) with hexane : diethylether (50:50) as eluent at a flow-rate of 3 ml/min and detection at 328 nm. The retinylester fraction eluted at 4 min. The entire fraction was collected and the solvent was evaporated by a stream of nitrogen. Then 1.5 ml 6% KOH (w/v) in methanol was added to the residue and the esters saponified by a 20 min incubation at room temperature. Out of a KOH concentration range (2 to 6%) and a range of incubation times (10 to 90 min)

optimal conditions were destillated: complete saponification could be achieved in 10 min with 6% KOH, and therefore routinely, saponification times of 20 min were employed to be on the safe side. At the end of the incubation, 2 ml hexane and 1 ml water were added and after vigorous mixing the two phases were separated by centrifugation (1-2 min; 7000 x g). The organic upper layer was collected and the lower layer re-extracted with 1 ml hexane. After evaporation of the solvent by a stream of nitrogen, the residue was dissolved in hexane/dioxane and the isomeric composition of the resulting retinols was determined by HPLC as described above (section 2.1.2). Although, in vivo only all-trans and 11-cis isomers should be present, a steady 10-15% of 13-cis isomers was always detected. This probably represents a saponification artefact.

2.1.4.2. Isomeric composition of the retinaldehyde fraction.

The retinaldehyde fraction was separated from the other retinoids by semi-preparative HPLC as described above (section 2.1.4.1). The retinaldehyde fraction eluted at 6 to 8 min. After evaporation of the solvent, the retinaldehyde fraction was reduced by adding excess sodium borohydride in methanol and incubating for 3-5 min at room temperature (Bridges, 1976). The resulting retinols were extracted and processed as described for the retinylester fraction. Again 13-cis isomers were detected, but were now probably already introduced as a side effect of retinoid extraction (Groenendijk et al., 1980a). Control experiments with all-trans retinaldehyde or 11-cis retinaldehyde did not present any evidence for artificial isomerization during reduction.

2.1.5. RETINOID SEPARATION BY HPLC WITH GRADIENT ELUTION.

The isocratic HPLC mode for the analysis of retinoids described in section 2.1.2 was used in experiments described in chapters 3, 4 and 5. For experiments in chapter 6, distribution of ^{3}H-label over the retinoid fractions had to be analyzed as well. Since the bulk of the label (ca 90%) appeared to be incorporated into retinylester baseline separation of the retinaldehyde from the retinylester fraction is required in order to prevent tailing of label from this fraction into the retinaldehydes. Although isocratic elution proved

to be suitable for retinoid quantification by peak-area integration, it did not sufficiently resolve these fractions to allow reliable analysis of the label incorporation into the retinaldehyde fraction. In order to improve the separation between retinylester and retinaldehyde, a gradient elution was developed with iso-octane/dioxane cocktails as eluens (iso-octane is recommended for gradient elution instead of hexane, due to lower degree of compressibility). Since performance of retinoid analysis did not alter, only the gradient profile and retention times will be described.

Gradient elution was under microprocessor control (LKB 2152 HPLC controller) which commanded a LKB 2150 gradient pump with detection at 328 nm (Kratos analytical Spectroflow 757 absorbance detector). The elution profile started with 0.3% dioxane during 3 min, then a gradient of dioxane (0.3 - 2.5%) in 5 min, after 4 min followed by a gradient of dioxane (2.5 - 7%) from 12 to 16 min and a steady dioxane level of 7% up to 25 min. At the end of each HPLC run the column was re-equilibrated with 0.3% dioxane. Detection limit: 0.05 pmol/all-trans retinol per HPLC run. Retention times are given in table 2.3.

Table 2.3. RETINOID SEPARATION BY ISOCRATIC OR GRADIENT HPLC

RETINOID	RETENTION TIME[a] ISOCRATIC MODE	GRADIENT MODE
retinylester	1.8	3
retinaldehyde		
11-cis (+13-cis)	3.3	8.7
9-cis	3.9	9.6
all-trans	4.3	11
syn all-trans retinaloxime	9	17.5
retinol		
11-cis	12	19.5
13-cis	13	20.0
all-trans	16	22.6

a: retention time in minutes

2.1.6. CONCLUSIONS.

Based on the procedure for extraction and analysis of retinoids from retina and rod outer segments, described by Groenendijk et al. (1980a), a rapid, reproducible and sensitive method was developed to extract and analyse (detection limit 0.05 to 0.2 pmol).

Although, complete resolution of all ocular retinoids and their isomers could only be achieved in a single run for retinols, saponification of the retinylester fraction (chapter 5 and 6) and reduction of the retinaldehyde fraction (chapter 6), allowed subsequent determination of their isomeric composition in a second run as the corresponding retinols.

2.2 DETERMINATION OF CELL DENSITY.

Isolation of bovine retinal pigment epithelium cells via the perfusion technique (chapter 3) yields clusters and sheets of RPE cells, which considerably vary in size and may contain from 10 to over 500 cells. This presents serious problems in determining cell densities by means of a haemocytometer or a particle counter like a Coulter Counter. Therefore the cell density was determined by means of a DNA assay using the weakly fluorescent dye DAPI which forms a strongly fluorescent complex with ds DNA of high molecular weight (Kapuscinski and Skoczylas, 1977). The assay was carried out according to Mier et al. (1982).

2.2.1. MATERIALS.

DAPI (4',6-diamidino-2-phenylindole-2-HCl) was obtained from Boehringer Mannheim GmbH (Mannheim, W-Germany) and high molecular weight double-strand DNA (human) was a generous gift of dr. J.A. Schalken, Dept of Urology, Nijmegen.

2.2.2. DETERMINATION OF CELL DENSITY BY MEANS OF A DNA ASSAY.

The conditioned media,in which the RPE cells were cultured or incubated (short-term incubation) affect the fluorescence intensity. Therefore an aliquot (50 µl) of a cell suspension was first diluted in 0.5 ml of perfusion buffer (an isotonic salt solution, for composition see section 3.2.1) and centrifuged

(Eppendorf 5414 centrifuge; 5 - 10 sec; 8,000 x g). Then, the cells were lysed by addition of 0.5 ml 10mM NaCl and sonicated on ice with a Branson B12 sonifier equipped with a microtip (10 Watt output) to release the DNA from the nucleus. Maximal DNA extraction from bovine RPE was obtained by at least 2 sonication bursts of 10 sec (see fig. 2.2). The supernatant obtained following subsequent centrifugation (5 - 10 sec; 8,000 x g) could either be used directly for the DNA assay, or kept overnight at 4°C.

A calibration curve in duplo (0.5 - 4.0 µg DNA/ml) of high molecular weight DNA and three dilutions in duplo of the RPE sample were prepared in 10 mM NaCl. For the assay, 500 µl of DAPI solution (25 ng/ml in 10 mM Tris-HCL, pH 7; just before use diluted 1000 times from a stock solution in dimethylsulfoxide kept at -20°C) was added to 50 µl of DNA solution. Although the resulting fluorescent complex was stable for at least 8 hr, usually the fluorescence was measured immediately by means of a Shimadzu Spectrofluorophotometer RF 150 (excitation wavelength: 360 nm, slit 20 nm; emission: 450 nm, slit 40 nm). Cell densities were calculated by regression analysis assuming 6 pg DNA/bovine somatic cell (Sober, 1970). Detection limit was ca 10,000 cells.

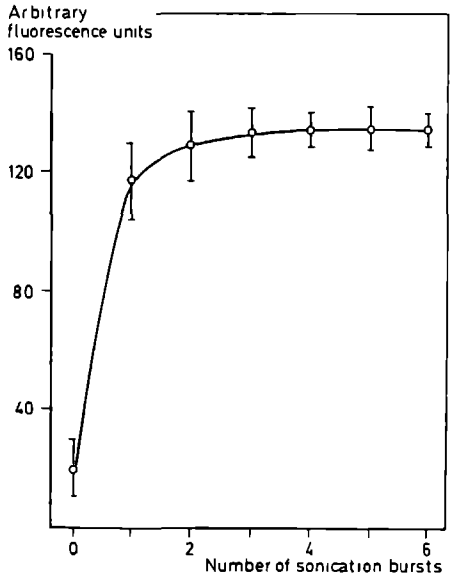


Fig. 2.2.
Release of DNA from bovine retinal pigment epithelium cells by sonication.
The amount of DNA released from bovine RPE cells (20,000 RPE cells) by sonication on ice with a Branson B12 Sonifier equipped with a microtip (output: 10 Watt) is expressed as arbitrarial fluorescent units obtained by the DNA assay. Two sonication bursts of 10 sec already yielded maximal DNA release. Routinely three sonication bursts were applied.

2.3. ELECTRO-IMMUNOBLOTTING.

The combination of SDS-PAGE, which resolves proteins by their Molecular Weight and immunotyping provides a powerful tool do detect or recognize specific proteins within a large population. This approach was used in the studies described in chapter 4 (presence of cellular retinol binding protein and/or cellular 11-cis retinoid binding protein in cultured RPE cells) and in chapter 7 (detection of proteolytic fragmentation pattern of opsin in RPE cells).

2.3.1. MATERIALS.

Swine anti-rabbit immunoglobulin, rabbit anti-mouse immunoglobulins, PAP (rabbit) soluble complex of horse radish peroxidase and rabbit anti-peroxidase immunoglobulin were obtained from Dakopatts (Glostrup, Denmark). 4-chloro-1-naphtol was obtained from Sigma Co (Rochester, New York, USA). Nitrocellulose paper (BA 85 membrane filter 0.45 µm) was obtained from Schleicher and Schuell (Dassel, W-Germany) and the low molecular weight calibration kit from Pharmacia (Uppsala, Sweden).

2.3.2. PROCEDURE.

2.3.2.1. Preparation of retinal pigment epithelium cells for polyacrylamide gel electrophoresis.

To a cell sample of 50 µl (maximally 0.5 x 10^6 RPE cells), 0.4 volume of 10% (w/v) sodium dodecyl sulfate (SDS) solution was added. This mixture was left to dissolve at room temperature for 10 min under repeatedly vigorous mixing, whereupon a freshly prepared solution of dithioerythrol (30mg/ml in Tris-HCl; pH 6.8) was added to a final concentration of 3 mg/ml. Following a second 10 min incubation at room temperature under repeatedly mixing, a Tris-glycerol-BPB cocktail (pH 6.8) was added to a final concentration of 50 mM in Tris-HCl, 10% in glycerol, and 0.005% in bromophenolblue. The resulting solutions are directly used for analysis by gel-electrophoresis.

2.3.2.2. SDS polyacrylamide gel electrophoresis.

A discontinuous gel system, modified after Laemmli (1970), was used. All solutions contained 0.2% SDS (w/v). Linear polyacrylamide gradient (12% - 22%) slab gels (0.7 mm thickness) were prepared form a stock solution of 40% (w/v) acrylamide and 1.07% (w/v) bis-acrylamide. The separation gel was prepared from a 12% acrylamide solution in 0.4 M Tris-HCl and 3.5% glycerol (pH 8.8) with a 22% acrylamide solution in 0.4 M Tris-HCl and 17.5% glycerol (pH 8.8) using a simple linear gradient former. The separation gel was left to polymerize overnight at room temperature. A stacking gel of 4% acrylamide in 0.15 M Tris-HCl (pH 6.8) was used to concentrate the samples before entering the separation gel and was applied on top of the separation gel after it had polymerized. The gels were run at 7.5 mA per gel during stacking and then at 15 mA per gel during separation for 5 to 6 hr under cooling by tap water. The electrode buffer contained 200 mM glycine and 25 mM Tris with in addition 0.1% SDS in the cathode buffer. After the end of the run, the gels were either prepared for electroblotting or fixed for two hr in isopropanol/acetic acid/water (25:10:65) and stained for protein with Coomassie blue R-250.

2.3.2.3. Transfer of proteins from the gel to nitrocellulose paper by means of electroblotting according to Burnette (1981).

The proteins separated by gelelectrophoresis are transferred electrophoretically during 16 hr at 4^{o}C to a sheet of nitrocellulose (BioRad power supply 160/1.6 at 60 Volt) in blotting buffer (25 mM Tris, 200 mM glycine, 20% methanol).

2.3.2.4. Visualization of antibody-antigen complexes.

Subsequent to electro blotting, the nitrocellulose sheet with the transferred proteins was rinsed with distilled water followed by incubation in a gelatin solution (blocking) in order to prevent aspecific antibody binding to nitrocellulose. Hereto the sheet was soaked in 3% gelatin in TBS (20 mM Tris-HCl and 500 mM NaCl; pH 7.5) and incubated at 37^{o}C for 2 hr. Following rinsing with excess TBS (3 x 10 min; room temperature), the nitrocellulose sheet was immersed in a solution of specific antibodies, diluted to a suitable concentration in 1% gelatin-TBS, and incubated overnight at room temperature. After rinsing with excess TBS (3 x 10 min;

room temperature), the sheet was incubated for 1 hr at room temperature with swine anti-rabbit immunoglobulins (diluted 1/100 in 1% gelatin-TBS) under gently swirling and again rinsed with excess TBS (3 x 10 min). In case of the monoclonal antibody, a 'bridge-reaction' with rabbit anti-mouse (1/100 in 1% gelatin-TBS) was performed first. Subsequently, the nitrocellulose blot was incubated for 1 hr at room temperature with rabbit peroxidase anti-peroxidase (PAP) (diluted 1/500 in 1% gelatin-TBS) under gently swirling.

The antibody complexes were detected through the bound peroxidase activity by incubation with a freshly prepared cocktail of 5 volumes of 0.02% hydrogenperoxide (v/v) in TBS and 1 volume of 0.3% (w/v) 4-chloro-1-naphtol in methanol. Peroxidase activity revealed itself within 1 - 5 min by a purple-blue precipitate. The reaction was stopped by rinsing the blot with distilled water in order to remove the substrate. After drying the blots can be stored in darkness.

2.3.2.5. Molecular markers.

A lane with molecular weight markers was also cotransferred to nitrocellulose by electro blotting. This lane was not processed for immunotyping but cut off from the nitrocellulose blot and the marker proteins were visualized by staining with amidoblack. Hereto, the piece of blot with marker proteins was soaked for 2 to 3 min in an 0.1% solution of Amido Black in a mixture of 45% methanol, 10% acetic acid and 45% distilled water. The blot was then de-stained with water until background staining was sufficiently low (ca 10 min).

A NEW ISOLATION PROCEDURE FOR RETINAL PIGMENT EPITHELIUM.

Adapted from:
Ad M.M. Timmers, Edward E. Dratz, Willem J. de Grip and Frans J.M. Daemen.

Invest. Opthal. Vis. Sci. (1984) 25: 1013-1018

3.1 INTRODUCTION.

As described in chapter 1, the retinal pigment epithelium is essential for proper maintenance and function of the vertebrate photoreceptor cells. It stores and recycles the retinal chromophore of the visual pigment and helps maintain the proper length of the outer segment.

Research on retinal pigment epithelium cells has been hampered strongly by the fact that "it is exceedingly difficult to prepare RPE cells with intact plasmamembranes" (Bridges, 1984). The most commonly used isolation techniques for RPE cells use eyecups from which the anterior part, the vitreous and the retina have been removed. The RPE cells then are collected either by brushing (Berman and Feeney, 1976) or harvested after preincubation with a proteolytic enzyme (Mannagh et al., 1973; Edwards, 1977). However, both these techniques approach the apical side of the RPE cells, whereas the attachment sites are at the base of the cells at Bruch's membrane. The RPE cells form the blood retinal barrier at the choroidal side (fig 3.1). As is usual, this epithelial cell layer contains an elaborate complex of tight junctions; solutions applied to the apical side cannot reach the attachment site of the cells at Bruch's membrane without breaking cells and/or tight junctional complexes. Consequently, mechanically detaching the RPE cells by brushing results in a large percentage of broken cells with concomitant release of cytoplasmic components (Saari et al., 1977).

Calcium and magnesium are thought to play an important role in the attachment of many cell types to basement membranes. Cardiac perfusion of rats leads to detachment of largely intact RPE cells (Katz, Farnsworth, Parker and Dratz, unpublished observation). The rat RPE is a small tissue and provides very limited amounts of material. Therefore, we have developed a perfusion technique for isolated bovine eyes using a buffer free of divalent cations. Under optimal conditions, this technique provides a high yield of intact bovine RPE cells.

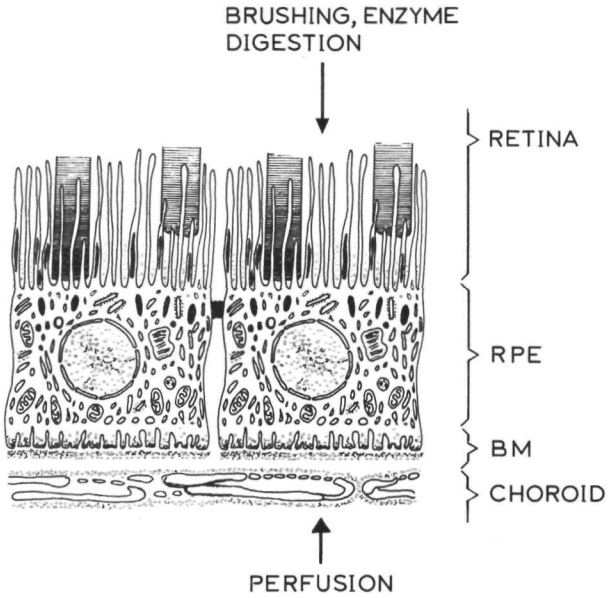


Fig 3.1
Approach of isolation techniques.
Cross section through posterior part of the eye illustrating the anatomy and various approaches to isolate retinal pigment epithelium cells. The pigment epithelium cells are connected by tight junctions. BM = Bruch's membrane.

3.2 MATERIALS AND METHODS.

3.2.1. MATERIALS.

Perfusion buffer (Ca-Mg-free HANKS EDTA) (Heller and Jones, 1980): 137.8 mM NaCl; 5.4 mM KCl; 0.3 mM Na_2HPO_4; 0.4 mM KH_2PO_4; 2 mM EDTA; 5.5 mM glucose; 10 mM HEPES (pH 7.4). Didansylcystine was obtained from Sigma Chemical Company (St. Louis, Missouri, USA). The buffers were sterilized (by autoclavation or by filtration).

3.2.2. METHODS.

3.2.2.1. Perfusion of the eye.

Bovine eyes were enucleated as soon as possible after the death of the animal thereby keeping the optic nerve at least 2 cm long. The eyes were put immediately into a light tight container. At the laboratory, trimming of excessive fat and muscle tissue under dim red light was followed by wrapping the eyes in aluminium foil leaving the optic nerve with the central ophthalmic artery

accessible. The wrapped eyes were kept in a dark container at 8 - 10°C.

The perfusion buffer reservoir was positioned 100 - 120 cm above the eye (fig 3.2), to which it was connected by cannulating the central ophthalmic artery with a blunt 21-gauge needle. The central ophthalmic artery, running along the optic nerve and supplying the entire eye, was identified easily by its translucent white color and the blood clot at its end. It could be distinguished further from surrounding fat tissue by pulling gently with forceps, which ruptured the fat tissue but not the artery. The artery was picked up with two small forceps (5SA, Technical Tools, Rotterdam, The Nederlands), pulled over the blunt needle and tied with a suture. The wrapped eye was perfused with ice-cold perfusion buffer for 13 - 17 min.

Routinely, the eyes were processed within 2 hr after death of the animal. Under those conditions less than 5% of the eyes had to be discarded for failure to perfuse.

3.2.2.2. Isolation of the retinal pigment epithelium cells.

The perfusion was ended by disconnecting the artery from the needle and the eye was unwrapped under normal fluorescent

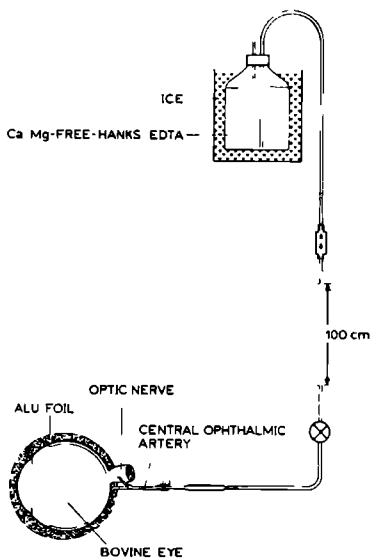


Fig 3.2
The perfusion set up.
Semi-diagrammatic drawing showing the set-up for perfusion of the bovine eye, adapted from the perfusion system for cat eyes (J. M. Thijssen, personal communication).

illumination. An incision was made posterior to the ora serrata and the anterior part was removed. Special care should be taken when removing the retina. The retina should not slide over the RPE layer, but should come out straight, so as to avoid considerable loss of RPE cells. This was done best by carefully detaching the retina at its edge and when it was about to dislodge by turning the eyecup so as to allow the retina to release by gravity. The retina then was cut off as close to the optic disc as possible.

The RPE cells were dislodged from Bruch's membrane by means of a gentle jet of perfusion buffer (culture medium, chapter 4, or incubation buffer, chapter 5) from a syringe equipped with a hypodermic needle (i.d. 0.4 mm). The harvested cell suspension was centrifuged at 40 x g (0^{o}C, 10 min), which should leave a clear supernatant. The sedimented cells were washed once with a large volume and resuspended in a small volume of the desired buffer. A sample was taken for determination of cell density, the rest was processed immediately for further analysis.

3.2.2.3. Retinoid analysis.

Retinoid extraction and analysis was performed as described in chapter 2 (section 2.1.2 and 2.1.3).

3.2.2.4. Determination of cell density.

Cell density was determined by means of a DNA assay carried out as described in chapter 2 (section 2.2.2).

3.2.2.5. Plasmalemma integrity: exclusion of didansylcystine.

The fluorescence quantum yield of didansylcystine increases about 20-fold when it is bound to membranes. This property had been used to check the plasmalemma integrity of frog photoreceptor cells (Yoshikami et al., 1974). In case of an intact plasmalemma, didansylcystine penetrates only slowly and the disc membranes shows no appreciable fluorescence. The suitability of didansylcystine as a probe for testing RPE plasmalemma integrity is apparently due to the high membrane density in RPE's cytoplasm (Katz, Farnsworth, Parker and Dratz, unpublished observations). When 50 µl of a cell suspension (1 - 2 10^{6} RPE cells/ml) (fig 3.3A) was mixed gently with an equal volume of a freshly prepared solution of didansylcystine (0.25 mg/ml) and examined immediately under a

fluorescence microscope (high pressure mercury lamp excitation light filtered through a Schott BG 12 filter), two cell populations could be observed: those that clearly showed fluorescence above the background and those that did not stain at all (fig 3.3B). The percentage of cells that showed fluorescence was constant for at least 5 min. Disruption of the plasmalemma by addition of detergent (0.5% hexadecyltrimethylammonium bromide (CTAB) or ethanol (70%) rendered 100% of the cells fluorescent within 15 sec. It was inferred that a relation existed between uptake of fluorescent dye and plasmalemma integrity.

The percentage of cells that did not take up the fluorescent dye was estimated and taken as a measure for the relative number of viable cells.

3.2.2.6. Plasmalemma integrity: retention of cellular retinol binding protein.

Determination of cellular retinol binding protein was carried out with the Lipidex micro assay as described in the appendix.

3.2.2.7. Ultrastructure.

Isolated RPE cells were sedimented (10 min; 40 x g; 4°C), resuspended in modified Karnovski's fixative (2% paraformaldehyde, 2.5 % glutaraldehyde, 0.1 M sodium cacodylate, pH 7.4) and incubated at room temperature for 2 hr. Subsequently, the fixated cells were left to sediment (10 min, 1 x g, room temperature), resuspended in 2% low temperature gelling agarose in 0.1 M sodium cacodylate (pH 7.4) and sedimented again (5 min, 200 x g, room temperature). The agarose was then left to solidify (0.5 - 1.5 hr, 4°C). The agarose embedded RPE cells were post-fixed with 2% OsO_4 (1 hr), dehydrated with graded increasing ethanol concentrations (30% to 100%), and immersed in propylene oxide (2 x 15 min). Embedding in Epon 812 (5A:5B) was initiated via propylene oxide/Epon 812 cocktails and finally followed by 100% Epon 812. Polymerization of Epon 812 was carried out at 60°C (3 days).

Ultrathin sections (silver: 60-80 nm) were cut with a diamond knife (Diatom, Schwitzerland) and subsequently uranyl acetate (30 min) and lead citrate (15 min) according to standard procedures and examined with a Philips EM 300 or EM 301 transmission electron microscope.

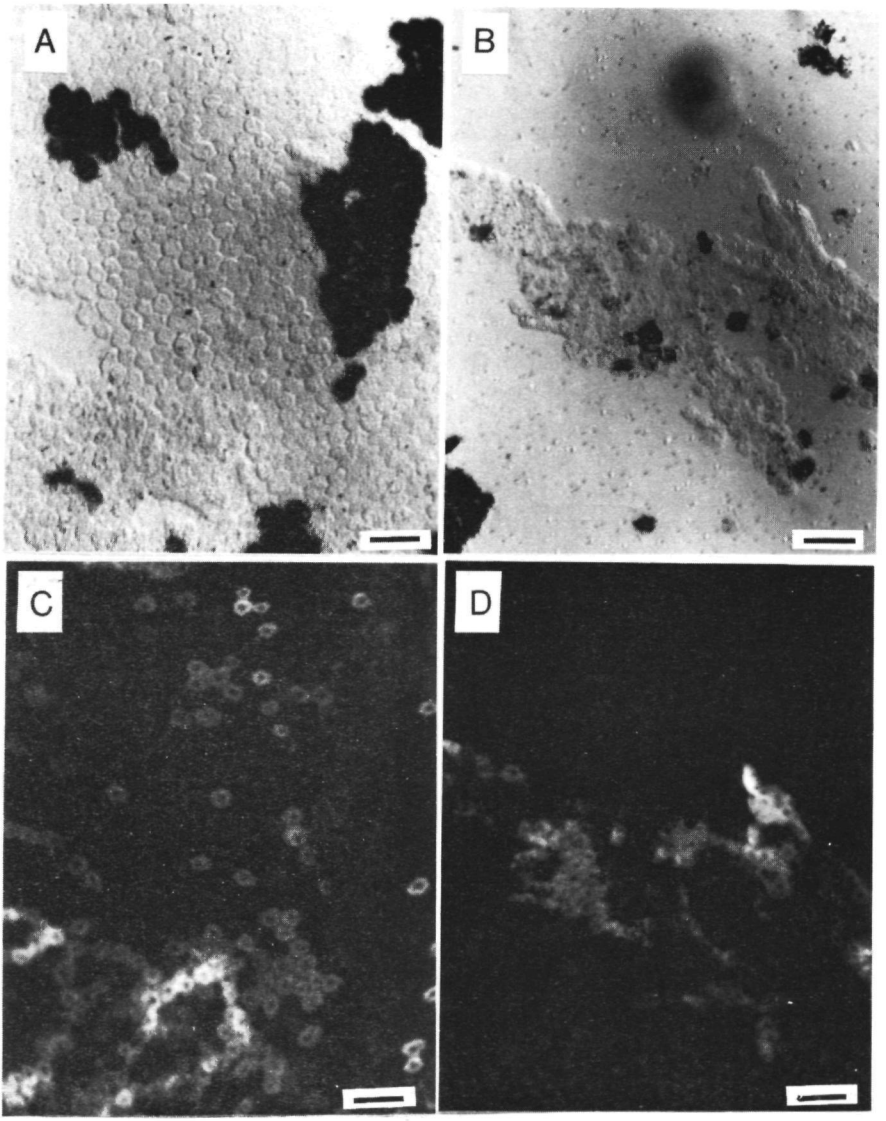


Fig 3.3
Plasmalemma integrity: exclusion of didansylcystine.
Phase contrast microscopy (A, C) of isolated RPE cells and analysis of integrity of plasmalemma with didansylcystine fluorescence (B, D). A, B: sheet of cells, typically obtained via the perfusion technique, largely showing amelanotic cells. C, D: isolated small cluster of cells obtained by the brushing technique (for a more typical brushing preparations see fig 3.4B). Leaky RPE cells show either strong or medium fluorescence intensity upon incubation with didansylcystine. We have not investigated whether a relation exists between fluorescence intensity and extent of cell damage. Only non-fluorescent cells have been counted as intact. Bar represents 50 um.

3.3. RESULTS AND DISCUSSION.

3.3.1. OPTIMIZATION OF THE PERFUSION TECHNIQUE.

The perfusion technique was optimized with respect to the following variables: (i) conditions of storage of the eye after removal from the animal; (ii) temperature of the perfusion buffer; (iii) dark/light adaptation of the isolated eye; and (iv) duration of perfusion. Parameters taken into account were: number of cells isolated per eye and percentage of intact cells.

In agreement with Feeney-Burns and Berman (1982), the best results were obtained when the eyes were removed immediately after death of the animal, allowed to cool to room temperature in the dark and then cooled to 8 - 10°C in the laboratory. When stored at lower temperatures (eg. on ice), many RPE cells adhered to the retina, which lead to considerable loss in yield. Routinely, the eyes were not stored for longer than 2 hr.

In agreement with Zauberman (1979), light adaptation during storage and/or perfusion induced a stronger adhesion between RPE and rod outer segments, resulting in considerable loss of RPE when the retina was removed. Hence the eyes were stored in a light-tight container and wrapped in aluminium foil under dim red light before being connected to the perfusion supply. The latter had to be done under normal laboratory light conditions, since the central ophthalmic artery was difficult to locate in dim red light.

Little effect was noted when the temperature of the perfusion buffer was varied between 0°C and 10°C. Routinely, the buffer was cooled on ice. The duration of the perfusion proved to be rather critical. Within the first 10 min, the divalent cation depletion was apparently not sufficient to weaken the adhesion of the RPE to Bruch's membrane, as shown by low yields of intact cells. Routinely, a perfusion time of 15 min was employed, but 20 min has been used without obvious negative effects.

3.3.2. CELL YIELD AND INTEGRITY OF THE ISOLATED RETINAL PIGMENT EPITHELIUM CELLS.

The perfusion technique and brushing approach were compared with respect to cell yield and integrity of the isolated RPE cells. The

integrity was evaluated along the following parameters: didansylcystine exclusion, retaining of all-trans retinol and cellular retinol binding protein and by the ultrastructure of the isolated cells.

3.3.2.1. Yield and exclusion of didansylcystine.

Both purity (fig. 3.4) and yield of intact cells improved significantly by applying the perfusion technique (table 3.1). Of the cells isolated by perfusion (fig. 3.3A,B), 70 - 80% did not take up didansylcystine, while almost none of the cells isolated by brushing (fig. 3.3C,D) presented a permeability barrier the fluorescent dye.

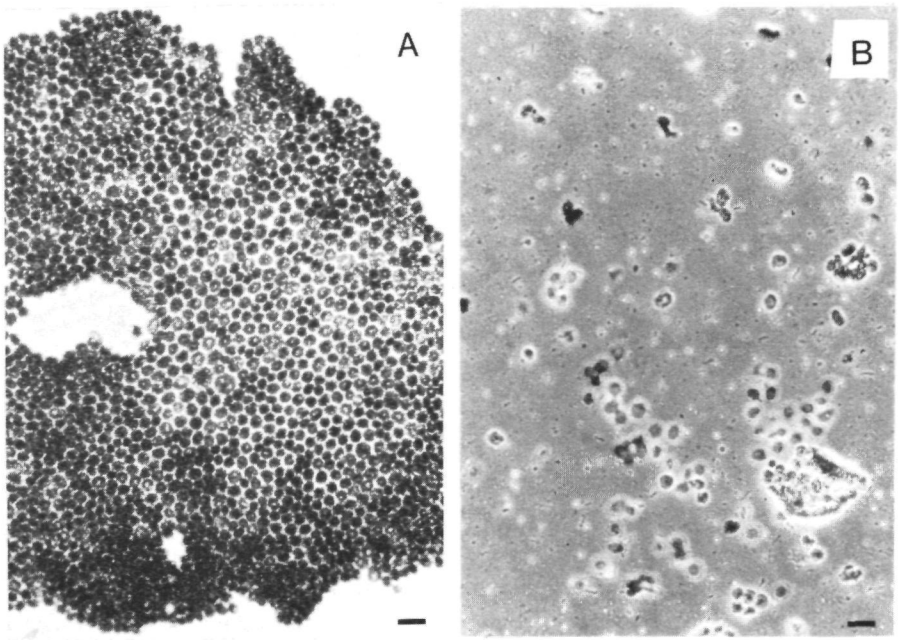


Fig 3.4
Retinal pigment epithelium cell preparations.
Typical examples of RPE preparations obtained by two isolation techniques. A: phase contrast micrograph of a sheet of RPE typically isolated from a bovine eye with perfusion, largely showing melanotic cells. Note the lack of contaminating rod outer segments (ca 5% on an outer segment to cell basis). B: Isolated cells typically obtained by the brushing technique. Note the contamination with rod outer segments. Bar represents 50 µm.

Although dye exclusion indicates a high percentage of intact cells, we also examined parameters directly related to vitamin A metabolism: cellular retinol binding protein and all-trans retinol. The retention of a small cytoplasmic protein like cRBP (M_r 15 kD) should be a reliable indicator for the percentage of the cells that retain cytoplasmic proteins. Since all-trans retinol represents the endogenous ligand of cRBP, a similar distribution as cRBP is to be expected.

2.3.2.2. Retention of cellular retinol binding protein and all-trans retinol.

Determination of the percentage of intact cells by their cRBP and retinol content requires information on their absolute content. By brushing a quantitative yield of RPE material can be obtained and this preparation was taken as calibration reference. After removal of the retina, the eyecup was rinsed with perfusion buffer and subsequently the RPE cells were brushed out. This mixture of intact and broken RPE cells was used to determine the absolute amount of all-trans retinol (110 $\pm$ 13 pmol/10^6 RPE cells; n=3) and of cellular retinol binding protein (140 $\pm$ 25 pmol/10^6 RPE cells; n=3). It should be kept in mind, however, that this preparation was probably contaminated to some extent by interphotoreceptor matrix (containing the interphotoreceptor retinoid binding protein) and rod outer segments.

The percentage of these compounds retained by RPE cells, isolated either via perfusion or by brushing, is given in table 3.1. The results fully agree with those obtained by the dye exclusion technique: about 80% of the cells isolated via perfusion retain cRBP, a small cytoplasmic protein. These findings emphasize that the perfusion technique largely yields intact cells. The all-trans retinol analysis also indicate that the perfusion technique is superior but unexpectedly by a much smaller margin. Apparently, all-trans retinol does not only occur bound to cRBP, as is evident in the brushed RPE cells (20 pmol all-trans retinol versus 3 pmol cRBP). Note however that the retinol level retained by the 'perfusion' cells (60 pmol/10^6 cells) is about half the cRBP level retained by the same population (120 pmol/10^6 cells). This level of saturation has been reported for cRBP in other tissues as well (Chytil and Ong, 1984). Probably the excess of

Table 3.1. YIELD AND INTEGRITY OF RETINAL PIGMENT EPITHELIUM CELLS ISOLATED BY PERFUSION OR BRUSHING.

	PERFUSION	BRUSHING
YIELD:		
cells/eye [1]	1 - 2 x 10^6	0.5 - 1.5 x 10^6
CELL INTEGRITY:		
dye exclusion [2]	70 - 80%	< 5%
cells retaining:		
cRBP [3]	85 ± 6%	2 ± 1%
all-trans retinol [3]	55 ± 7%	20 ± 4%
ultrastructure	+	-

1: total number of RPE cells: 5 ± 0.5 x 10^6 cells/bovine eye (n=3).
2: number of cells counted ca. 5000 (perfusion) and ca 1500 (brushing).
3: number of determinations: 3.

retinol in 'brushing' preparation, which is partly retained by the cell pellet originates in contamination (ROS, interphotoreceptor matrix; cf fig 3.4B).

3.3.2.3. Ultrastructure.

Mechanical isolation of RPE cells has been demonstrated to cause serious ultrastructural detererioration (Saari et al., 1977). In the final stage, the perfusion technique isolates RPE cells by mechanical means well. Although the biochemical evidence for non-leaky, integer 'perfusion' RPE cells is rather straight forward, the ultrastructural characteristics of the RPE cells isolated by perfusion and brushing were evaluated as well.

The ultrastructural parameters taken into account were: the morphological appearance of (i) nuclear membrane, (ii) cytoplasm and (ii) mitochondria. The RPE cells isolated by brushing (fig 3.5A) exhibited swollen and broken nuclear membranes, while the cytoplasmic content appeared to be lost to a large extent and showed swollen membraneous structures and disturbed rough endoplasmic reticulum. The mitochondria displayed a deterioriated morphology as well. The RPE cells isolated by the perfusion technique, on the contrary, exhibited excellent preservation of the

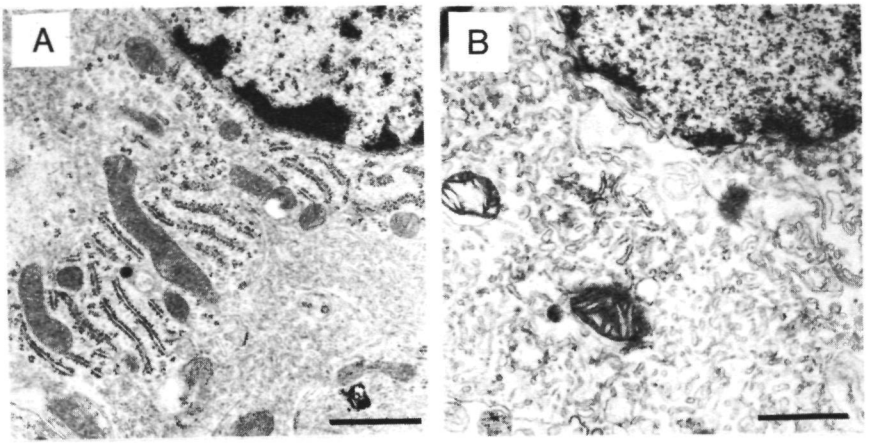


Fig 3.5
Ultrastructure of isolated retinal pigment epithelium cells.
Typical examples of RPE preparations obtained by two isolation techniques. A: the ultrastructure of RPE obtained by perfusion of the eye. B: cells isolated by the brushing technique. Bar represents 1 µm.

nuclear membrane, cytoplasm and mitochondria. These findings completely agree with the results of the biochemical evaluation.

3.4. CONCLUSIONS.

The perfusion method described, markedly improves the yield, purity and integrity of the isolated retinal pigment epithelium cells as compared to the previously published technique of brushing. RPE isolation by means of a proteolytic digestion yielded irreproducable amount of cells in our hands (0.05 - 3 x 10^6 cells per eye) and were not analyzed in as much detail. The perfusion technique affords a RPE cell preparation with minimal damage and high purity (ca 5% on an rod outer segment to cell basis). These cells could therefore present a very suitable substrate for metabolic studies on e.g. vitamin A metabolism, phagocytosis and other biosynthetic or regulatory mechanisms.

Chapter 4

RETINAL PIGMENT EPITHELIUM CELL CULTURE:

SUITABLE FOR FUNCTIONAL STUDIES?

4.1. INTRODUCTION.

The retinal pigment epithelium cell is an essential element in the physiological preservation of the photoreceptor cell (cf chapter 1). In the previous chapter we have described a procedure to isolate intact bovine RPE cells in fair quantities. However, biochemical studies on RPE require a regular supply of relative large amounts of material. Since, the limited availibility of freshly isolated RPE cells would consequently require a high frequency of cell isolation, it seemed logical to opt for an alternative, i.e. to maintain isolated RPE cells in culture. The advantages are obvious: large amounts of pure RPE cells in controlled environments, not influenced by components of retina nor choroid and available at any time. RPE cells of several species have been cultured succesfully. This includes chick (Newsome et al., 1974; Chader et al., 1975; Redfern et al., 1977; Middleton, 1977; Crawford et al., 1981; Turksen et al., 1983; Entani et al., 1986), rat (Edwards, 1977; Hall and Quon, 1981; Irons and Kalnins, 1984; Clark et al., 1984), human (Albert et al., 1972; Mannagh et al., 1973; Flood et al., 1980; Delmonte and Maumenee, 1981; Boulton et al., 1982; Hu et al., 1982; Oka et al., 1984), cat (Stramm et al., 1983), pig (Francois et al., 1971), rabbit (Nicolaissen et al., 1980; Tsukamoto and Ludwig, 1983) and cattle (D'Amico et al., 1982; Basu et al., 1983; Oka et al., 1984). However, their is a serious risk of loss of phenotypic characteristics in cell culture, which might be accompanied by loss of specific cell functions and activities (dedifferentiation), in particular since in vivo the cells hardly show any mitotic activity (at least in the human eye).

This chapter addresses the question whether RPE cells isolated by the perfusion technique, can be succesfully brought in culture and whether these cells, once in culture, maintain their highly differentiated status. This is a prerequisite if they are to be used for biochemical and cell biological studies. Possible occurrence of cell dedifferentiation in culture will be evaluated on a structural level, by monitoring cell morphology and ultrastructure, and on a functional level, by measuring some relevant activities in retinoid metabolism (cellular retinol binding protein, cellular 11-cis retinoid binding protein, retinol uptake and retinol esterification).

4.2. MATERIALS AND METHODS.

4.2.1. MATERIALS.

Minimum essential medium (MEM) was obtained from Flow Laboratories (Irvine, Scotland), HAM's F12 and Dulbecco's modified essential medium (DMEM) from Gibco Europe Limited (Renfrewshire, Scotland). Foetal calf serum was obtained from Flow Laboratories (Irvine, Scotland) and antibiotics from Gist-Brocades (Delft, The Netherlands). The tissue culturing flasks (25 and 75 cm^2), petridishes and six-well culture plates (10 cm^2) were obtained from Costar (Cambridge, Massachucetts, USA). Polylysine was obtained from Sigma Chemical Company (St. Louis, Missouri, USA), gelatin from Difco Laboratories (Detroit, Michigan, USA). Type IV collagen (isolated from bovine lung) was a generous gift from Dr J. Langeveld (Depart. of Biochemistry, University of Nijmegen, Nijmegen). Antisera directed towards cellular retinol binding protein (anti-cRBP) and cellular 11-cis retinoid binding protein (anti-cRAlBP) were a generous gift from Dr. J.C. Saari (Depart. of Ophthalmology, University of Washington, Seattle). Glutaraldehyde and paraformaldehyde were obtained from E.M. Science (Washington, USA) and Epon 812 from Merck AG (Darmstadt, W. Germany). Bovine serum albumin (BSA, fraction V) and trypsin inhibitor were obtained from Sigma Chemical Company (St. Louis, Missouri, USA). All other chemicals were of the highest grade available.

4.2.2. METHODS.

4.2.2.1. Cell isolation.

Isolation of bovine RPE cells was performed via the perfusion technique, as described in chapter 3.

4.2.2.2. Determination of cell density.

Cell density was determined by means of a DNA assay carried out as described in chapter 2 (section 2.2.2).

4.2.2.3. Cell culture.

Bovine PPE cells, isolated under the best possible sterile conditions from eyes of 1.5 to 2.0 years old animals, were

harvested in culture medium supplemented with 10 mM glutamine, 10 mM pyruvate, 10% FCS and antibiotics (penicilline: 100 I.U./ml; streptomycine: 50 µg/ml). The RPE cells were seeded in culture flasks or wells in a density of 3 - 5 x 10^4 cells/cm^2. Cell culture was performed at 37°C and, dependent on the medium, under 100% air (MEM and HAM's F12) or a humidified atmosphere of 5% CO_2/95% air (DMEM). The cells were kept for 3 days to allow them to attach to the bottom of the culture vessel. Subsequently, the cells, which were not firmly attached, were removed together with the culture medium by short agitation. The remaining cell density in this medium was determined via the DNA assay and used to calculate the percentage of cells which had attached. Routinely, the medium of the cell culture was refreshed two to three times a week.

After the cells had reached confluency, they were subcultured. Hereto the cells were detached by trypsin digestion solved in tyrode buffer (in mM, NaCl 137; KCl 3; Na_2PO_4 0.4; $NaHCO_3$ 12; glucose 10; EDTA 3) at 0.35 mg/ml for 3 - 5 min at 37°C; 0.1 to 0.15 ml/cm^2. The cells were collected by short agitation, pelleted (10 min at 900 x g; Hereaus Christ Minifuge GL; room temperature), washed by resuspension and centrifugation and finally resuspended in culture medium. The cells harvested from one culture flask were divided over 4 to 5 flasks. Nearly complete cell attachment (over 90%) was achieved in one to two days of subculture.

When cultured RPE cells were subjected to biochemical analysis, the cell cultures were rinsed twice with perfusion buffer (an isotonic salt solution lacking divalent cations, for composition see chapter 3, section 3.2.1) trypsinized and harvested in perfusion buffer containing trypsin inhibitor (0.2 mg/ml). The cells were then washed twice in perfusion buffer (10 min at 900 x g) and either used directly for analysis or stored as a pellet at -20°C.

4.2.2.4. Retinoid analysis.

Retinoid extraction and analysis was performed as described in chapter 2 (section 2.1.2. and 2.1.3).

4.2.2.5. Estimation of cellular retinol binding protein.

Determination of cellular retinol binding protein was carried out with the Lipidex micro assay as described in the appendix.

4.2.2.6. Electro-immunoblotting.

SDS-PAGE, electro-immunoblotting and visualization of antibody-antigen complexes was performed as described in chapter 2 (section 2.3). Antisera were diluted 50-fold (anti-cRBP) or 500-fold (anti-cRA1BP).

4.2.2.7. Ultrastructure.

A culture of RPE cells was rinsed with perfusion buffer and fixated during 2 hr with modified Karnovski's fixative (2% paraformaldehyde; 2.5% glutaraldehyde; 0.1 M sodium cacodylate; pH 7.4). The fixated cell culture was then rinsed with cacodylate buffer (0.1 M, pH 7.3) and post-fixated with 2% OsO_4 for 1 hr. The cells were then dehydrated in situ by means of series of graded ethanol solutions (30% to 100%) and embedded, via intermediate ethanol/Epon 812 cocktails (24 hr), in Epon 812 (4A:6B). followed by 100% Epon 812. Polymerization was started at relatively low temperature to ensure complete removal of ethanol ($30^{o}C$; 48 hrs). Polymerization capsules of which the bottoms were cut of were then pressed through the Epon 812 impregnated cells. The capsules were filled with Epon 812 and incubated at $65^{o}C$ for 3 days. The capsules with a piece of cell culture could then easily be removed from the bottom of the culture vessel. According to standard procedures, ultrathin sections were then cut, stained with uranyl acetate (30 min) and lead citrate (15 min) and examined with a Philips EM 300 or EM 301 transmission electron microscope.

4.3. RESULTS AND DISCUSSION.

4.3.1. OPTIMIZATION OF BOVINE RETINAL PIGMENT EPITHELIUM CELL CULTURING CONDITIONS.

Several media (MEM, HAM's F10 or F12 and DMEM) have been demonstrated to be suitable for cell culture of RPE cells. In order to improve cell attachment, cell growth or to slow down cell dedifferentiation, various substrates such as polylysine,

fibronectin or various types of collagen are commonly used to coat tissue culture vessels. Depending on the particular cell type different effects on the performance of the cells in culture have been observed. Many epithelial cells show preference for type IV collagen (a basement membrane component) above substrates of other collagen classes (Murray et al., 1979; Wicha et al., 1979; Terranova et al., 1980). In order to optimize culture conditions for bovine RPE cells both factors were evaluated: culture media (MEM, HAM's F12 and DMEM) in combination with a variety of coatings: type IV collagen (2 $\mu g/cm^2$, Terranova et al., 1980; Del Monte and Maumenee, 1981), polylysine (50 $\mu g/cm^2$, Kyristis et al., 1984), serum and gelatin (a collagen degradation product) (Del Monte and Maumenee, 1981). The following two selection parameters were applied: (i) percentage of cell attachment and (ii) time required to reach confluency in the primary culture. The percentage of attachment represents the percentage of the seeded cells which became firmly attached in 3 days. Confluency was estimated by light microscopy (phase contrast). The results are given in table 4.1. Surprisingly, the performance of bovine RPE cells in culture appeared rather to depend on the culture medium and secondary conditions (CO_2 incubator) than on the type of substrate. Further, the morphology of the RPE cells in the various culture conditions

Table 4.1. BOVINE RPE PERFORMANCE IN CULTURE.

CULTURE MEDIUM AND COATING	ATTACHMENT PERCENTAGE	DAYS TO REACH CONFLUENCY
MEM	30 - 40	15 - 20
+ collagen IV	30 - 40	15 - 20
HAM's F12	30 - 40	15 - 20
+ collagen IV	30 - 40	15 - 20
DMEM	50 - 60	10 - 16
+ collagen IV	70 - 80	10 - 16
+ polylysine	70 - 80	10 - 16
+ gelatin	70 - 80	10 - 16
+ FCS	70 - 80	10 - 16

Coating concentration: type IV collagen: 2.0 $\mu g/cm^2$; gelatine: 500 $\mu g/cm^2$; polylysine: 50 $\mu g/cm^2$. Collagen coating was carried out by slow evaporation of the collagen solvent (0.01 M acetic acid) in approximately 48 hr. Polylysine (0.05% w/v), gelatin (0.5% w/v), and FCS were incubated for 1 - 2 hrs at 37°C. Rest of the incubation mixture was sucked away. Number of experiments 5 to 10.

was very similar (see section 4.3.2.1). Based on practical considerations (minimal chance of contamination, number of manipulations), the following culture conditions were used routinely. Culture medium: DMEM (+ 10% FCS); substrate: coating with FCS (2 hr at 37°C); secondary conditions: 37°C under humidified atmosphere of 5% CO_2 and 95% air. Under these conditions the cells have a doubling time of 2 - 3 days and could be subcultured for up to 5 - 6 times.

4.3.2. CHARACTERIZATION OF RETINAL PIGMENT EPITHELIUM CELLS IN CULTURE.

The cell cultures of bovine RPE cells were evaluated according to cell morphology on a light- and electron microscopic level on the one hand and to some functional aspects on the other hand. To address the latter some properties were selected, which are related to the vitamin A metabolism (content of retinoid binding protein, retinol uptake and esterification).

4.3.2.1. Morphology and ultrastructure.

Morphology

The perfusion technique developed in chapter 3 for isolation of bovine RPE, yields sheets of RPE cell clusters of variable size. Such clusters attached spontaneously without requiring further desintegration into single cells by proteolytic treatment. Depending on their position in the clusters the cells displayed several morphological changes when brought into culture. Attachment of the cell clusters to the culture substrate was followed by spreading and formation of long slender pseudopod-like extensions, most obviously at the edge of the sheet. The cells in the center of such an 'island' tended to remain in a stationary phase, whereas mitotic activity seemed to be high at the edges resulting in rapid proliferation.

One of the most striking features was the loss of pigmentation. Even the pigmented RPE cells obviously could not switch on melanin biosynthesis upon 'induced' mitotic activity and the existing granules were diluted over the daughter cells. The typical hexagonal cell shape was still predominantly preserved present in the primary cultures, but the cells in subculture largely lost this

particular feature as well (fig 4.1A). RPE cells in subculture displayed a variety of shapes: in addition to polygonal epithelial-like cells (fig 4.1B, upper part), fibroblast-like spindle shaped cells growing in lanes (fig 4.1B, middle) as well as large sheet-like cells (fig 4.1B, lower) were observed.

Ultrastructure

On the ultrastructural level bovine RPE cells in vivo show microvilli and tight junctions at the apical side and infoldings at the basilar side. Rough endoplasmic reticulum and an abundance of mitochondria and membraneous structures are evident in the cytoplasm (fig. 1.5). The effects of cell culture on the ultrastructure of bovine RPE cells was examined in a confluent secondary culture (fig 4.2). The cell polarity as well as many characteristic cytoplasmic features appeared to have been lost completely. Further, the cells grew in several layers over each other and there was no evidence for the contact inhibition

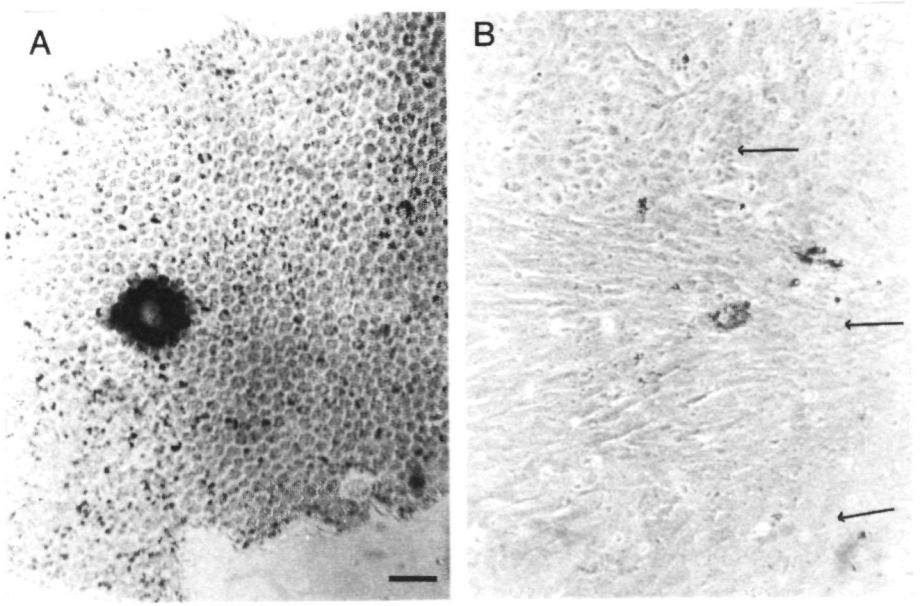


Fig 4.1
Morphology of RPE cells in culture.
Phase contrast micrographs of RPE cells freshly seeded (A) and in secondary culture (B). Note three major forms of bovine RPE in culture: hexagonal cells (upper arrow), spindle-shaped cells (middle arrow) and sheet-like cells (lower arrow). The bar represents 50 µm.

displayed in vivo. Only occasionaly tight junctions were detected. Probably, the slender processes between the cells represented extensions of cell bodies.

4.3.2.2. Functional characterization of retinal pigment epithelium cells in culture.

Retinoid binding proteins

Retinoid binding proteins play an essential role in the retinoid metabolism of the RPE cells. Two retinoid binding proteins have been detected in RPE: the cellular retinol binding protein (cRBP), which is common to many tissues, and the cellular 11-cis retinoid binding protein (cRAlBP), which appears to be unique to retina and RPE. The extend expression of these proteins in the cultured RPE cells would be a major indicator whether these cells present suitable substrate for studies on vitamin A metabolism in RPE.

The presence of the retinoid binding proteins was analyzed in a qualitative way by means of electro immuno blotting. In addition the cRBP content was quantitatively determined by means of the

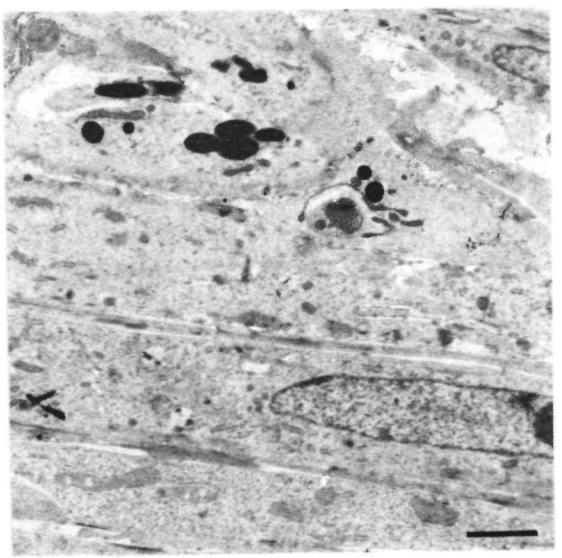


Fig 4.2
Ultrastructure of RPE cells in culture.
Bovine RPE cells in secondary culture. Note the multiple cell layers and the slender processes between cells. Supplementation of culture medium with retinol did not affect the ultrastructure. The bar represents 1 µm.

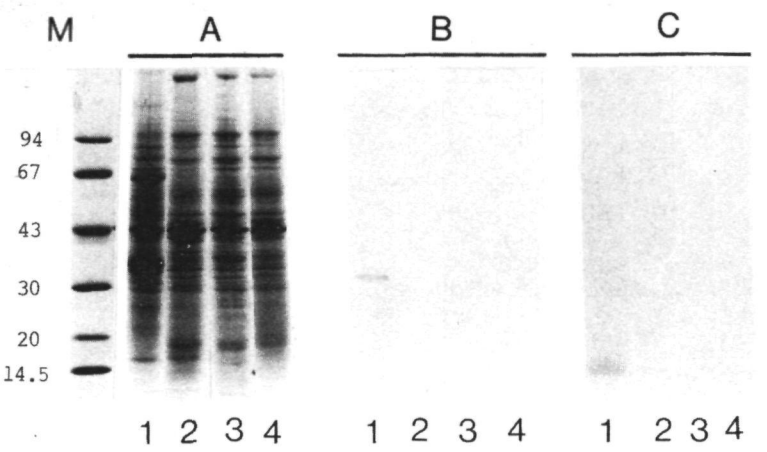


Fig 4.3.
Presence of retinoid binding proteins in RPE cell culture.
SDS-PAGE analysis of freshly isolated cells and cultured cells (A: coomassie brilliant blue staining) and subsequent electro immuno blotting and detection of cRAlBP (B) and cRBP (C) with specific antibodies. Lane 1 represents freshly isolated cells, lane 2 the primary culture after reaching confluency, lane 3 the secondary culture, lane 4 the tertiary culture. Per lane 30 x 10^4 cells were applied. Note the complete disappearance of cRAlBP and the strong decline of cRBP already in the primary culture. Lane M represents the molecular markers (MW x 10^{-3} kD).

Table 4.2. RETINOL UPTAKE AND PROCESSING IN RETINAL PIGMENT EPITHELIUM CELL CULTURE.[a]

CULTURE	NOT SUPPLEMENTED		SUPPLEMENTED[b]	
	RETINOL	RETINYLESTER	RETINOL	RETINYLESTER
PRIMARY	4	4	N.D.	N.D.
SECONDARY	< 2	< 2	45	28
TERTIARY	< 2	< 2	12	6

a: Retinoids are given in pmol/10^6 RPE cells. The experiment was carried out once. N.D: not determined.
b: Culture medium was supplemented with 0.4 µM retinol (ca 0.5 times the blood concentration in human) which is equivalent to ca 500 to 1000 pmoles/10^6 confluent cells.

Lipidex micro assay (fig 4.3 and table 4.2). The levels of both retinoid binding protein declined precipitously in the primary culture already. Cellular 11-cis retinoid binding protein was not detectable at all. Cellular retinol binding protein could still be detected, but its level dropped in the primary culture to approximately 10% of that in freshly isolated cells and dropped in subsequent subcultures even further to a fairly constant 5% of the original level.

Retinol uptake and processing

The RPE cells contain the retinoid supply of the retina as the retinylester and to that effect have a very active retinol uptake and esterifying activity with a large capacity. We investigated whether this activity was fully maintained in cell culture by supplementing the culture medium with 0.4 μM retinol, with BSA as carrier (Oka et al., 1984). Supplementation was started in the secondary and continued through the tertiary culture. The supplemented culture medium was refreshed every two days. After four days in tertiary culture the cells were harvested and assayed with respect to retinoid content (table 4.3). The presence of low amounts of retinol and retinylester (about 40% and 2% of the original level) demonstrates that the cultured cells are still able to take up and esterify retinol, but that this activity too is dramatically reduced compared to freshly isolated cells (cf chapter

Table 4.3 CONTENT OF CELLULAR RETINOL BINDING PROTEIN IN RPE CELL CULTURE.[a]

CELLS	NON SUPPLEMENTED	SUPPLEMENTED[b]
freshly isolated	120 ± 15 (3)	
primary culture	11 ± 3 (2)	
secondary culture	10 ± 3 (2)	5 ± 3 (2)
tertiary culture	6 (1)	5 ± 3 (2)

a: Values are given in pmol cRBP/10^6 RPE cells and represent a mean value of several determinations. Number of determinations are given between parentheses. cRBP measurements are performed with the Lipidex micro assay (cf appendix).
b: Culture medium was supplemented with 0.4 μM retinol.

5 and 6). No indication was obtained for isomerization or conversion of retinol into retinaldehyde.

4.3.2.3. Effect of retinol on differentiation.

The broad spectrum of biological activities of retinoids includes a role in differentiation of epithelial cells. Supplementation of the culture medium with all-trans retinol might therefore inhibit morphological and functional dedifferentiation. Hence, we investigated whether supplementation of the culture medium with 0.4 µM retinol, as described above, had a positive effect on preserving the ultrastructure and expression of cellular retinol binding protein. No effect whatsoever could however be detected (fig 4.2 and table 4.2).

4.4. CONCLUSIONS.

Bovine RPE cells, isolated via perfusion of the eye, appear to be easily able to survive in vitro under proper conditions and to form confluent cell cultures. They can be cultured in several media and on a variety of substrates. In comparison with literature data the bovine RPE cells, isolated via the perfusion technique, display a higher attachment efficiency (20% versus 70%), but further similar growth characteristics (doubling time of 2 - 3 days) and similar loss of pigmentation and morphology (Basu et al., 1983; Oka et al., 1984).

Cultured RPE cells have been considered to be suitable substrate for studies with respect to phagocytosis (Edwards and Bakshian, 1980; Hall, 1978). Unambiguous evidence that all elements of this process are completely retained by cells in culture has however never been presented. Only a few reports discuss vitamin A metabolism or related aspects in cultured RPE cells. Human RPE in culture has been shown to contain cellular retinol binding protein (Hu et al., 1982; ca 90% in primary culture and ca 25% in subculture, Blaner et al., 1985). Furthermore, incorporation of retinol, carried by BSA, and subsequently esterification has been demonstrated in cultured human RPE, allthough the fatty acid composition of the resulting retinylesters differed from that in vivo (Flood et al., 1983). The relative esterification level, which varied from 30 to 200% in the primary culture, decreased in

subcultures to ca 10%.

Bovine RPE, cultured under the conditions described above, exhibits a strong reduction in cellular retinol binding protein and cellular 11-cis retinoid binding protein (see above). Further, these cells retain retinol uptake and esterifying activity, but at a very reduced level. Supplementation of the culture medium with retinol does suppress neither morphological nor functional dedifferentiation. These findings are fully collaborated by a very recent report of Bridges et al. (1986), which describes complete loss of esterifying activity and of cellular 11-cis retinoid binding protein and a strong reduction in cellular retinol binding protein in their bovine RPE cell culture.

Both these results allow very definite conclusions: the vitamin A metabolism in cultures of bovine RPE cells is allmost completely suppressed under the conditions described. Hence, as yet no culture conditions have been found, where bovine RPE cells present a relevant substrate to investigate the vitamin A metabolism.

Studies on the vitamin A metabolism in RPE relevant to the visual cycle definitely requires RPE cells which still contain their entire retinoid metabolic machinery. Since the RPE cell culture does not satisfy these requirements, an alternative approach is required. In the following chapter the development of a short term incubation system for freshly isolated bovine RPE cells will be described which might represent a suitable alternative.

Chapter 5

FRESHLY ISOLATED BOVINE RETINAL PIGMENT EPITHELIUM CELLS: POTENTIAL FOR SHORT-TERM INCUBATION STUDIES EVALUATED BY THEIR ENERGY CHARGE AND RETINOID COMPOSITION.

5.1. INTRODUCTION.

The retinal pigment epithelium (RPE) cells form a monolayer of fragile cells, juxtaposed between the retina and choroid and tightly cemented onto Bruchs membrane. The RPE fulfills a crucial role in the vitamin A metabolism relevant to vision (the visual cycle).

The engagement of the RPE in the visual cycle has been well documented since 1878: 1. Regeneration of visual pigment does not occur in the isolated frog retina, but can be restored by recontacting retina and RPE (Kuhne, 1878), 2. Strong illumination (bleaching over 90% of the visual pigment) effectuates a retinol flux to the RPE. This flux is disturbed by disengaging the retina and the RPE, which causes retinol accumulation in the outer segments (Dowling, 1960), 3. Upon reinstalling a fully bleached bullfrog retina, which contains porphyropsin dorsally and rhodopsin ventrally, back onto the RPE after 180^{o} rotation and susequent regeneration the visual pigment distribution is reversed as well. This elegantly demonstrated that the RPE cells provide the chromophoric group or a precursor for the regeneration of bleached visual pigment (Reuter et al., 1971), and 4. In species where RPE cells have long microvilli, rhodopsin regeneration starts at the basal part of the rod outer segment, while it starts apically in species with short RPE extensions (Williams and Penn, 1985).

It has been recently demonstrated that the RPE takes up retinol both from the blood circulation and from the retina, and stores it as the retinylester (Defoe and Bok, 1983; Alvarez et al., 1981). The current model for the visual cycle (fig 1.7) places both hydrolysis of the retinylester and the oxidation of 11-cis retinol to 11-cis retinaldehyde in the RPE cell, whereas the isomerization of all-trans retinol to 11-cis retinol is placed in the Muller cells. This model is essentially based on the retinoid status of the isolated RPE cells and on localization and endogenous ligands of the ocular retinoid binding proteins (see table 1.2).

More detailed knowledge of the metabolic routes of the retinoids in the RPE cell and their regulation is required for better understanding of the contribution of the RPE to the visual cycle. However, this type of investigation requires a suitable in vitro system and has been strongly hampered by the fact that it proved

quite difficult to isolate large amounts of intact RPE cells. Recently we developed an isolation technique, (chapter 3, Timmers et al., 1984) based on perfusion of the bovine eye, which provides large amounts of intact RPE cells. In cell-culture these cells rapidly dedifferentiate (chapter 4 and Bridges et al, 1986), which renders a cell-culture system unsuitable for metabolic studies. Here, we report on the development of a short-term incubation system which allows metabolic studies on isolated RPE cells over a time period of at least 8 hours.

5.2. MATERIALS AND METHODS.

5.2.1. MATERIALS.

RPMI 1640 DM was obtained from Flow Laboratories (Irvine, Scotland) and supplemented with 10 mM glutamine and 10 mM pyruvate. Krebs Ringers contained (in mM): NaCl 119; KCl 3.7; $CaCl_2$ 2.5; $MgCl_2$ 1.2; KH_2PO_4 1.2; glucose 5.5; HEPES 20 (pH 7.4). Perfusion buffer contained (in mM): NaCl 138; KCl 5.4; Na_2HPO_4 0.3 KH_2PO_4 0.4; EDTA 2; glucose 5.5; HEPES 10 (pH 7.4). Petri dishes and culture plates were obtained from Costar (Cambridge, Massachuchetts, USA).

5.2.2. METHODS.

5.2.2.1. Cell isolation.

Isolation of bovine RPE cells was performed via the perfusion technique as described in chapter 3 (section 3.2.2.1 and 3.2.2.2).

5.2.2.2. Determination of cell density.

Cell density was determined by means of a DNA carried out as described in chapter 2 (section 2.2.2).

5.2.2.3. Cell incubation.

Incubation of the RPE cells was carried out in petri dishes (energy charge analysis) or 12-wells culture plates (retinoid analysis). Hereto, one volume of incubation medium was mixed with one volume of the isolated RPE cell suspension (energy charge analysis: 1.5 ml; retinoid analysis: 0.4 ml) to a final cell

density of 1 - 2 x 10^6 cells/ml. The cell suspension was then incubated at 37°C under a humidified atmosphere of 95% O_2 and 5% CO_2. At the end of the incubation the cells were collected by centrifugation (Hereaus Christ Minifuge GL, 10 min, 1000 x g, 4°C). The incubation wells were rinsed once with medium which was used to wash the cells as well. The final cell-pellet was either stored at -20°C or immediately processed for analysis.

5.2.2.4. (Ribo)nucleotide analysis for energy charge calculation.

Extraction and analysis of the (ribo)nucleotides was carried out according to De Abreu et al. (1982). In brief, the RPE cell-pellet (5 - 6 x 10^6 cells) was extracted by addition of 200 µl of icecold $HClO_4$ (1 M) under vigorous mixing (final concentration ca 0.4 M). After 15 min incubation on ice, the denatured proteins and membranes were precipitated (7000 x g, 10 min, 4°C), the supernatant was neutralized by addition of an icecold solution of 0.4 M KOH, 1 M KH_2PO_4 and a trace of phenol red and the resulting turbid solution was kept for 15 min at 0°C to precipitate $KClO_4$. Following centrifugation (7000 x g, 10 min, 4°C) the supernatant was either stored at -20°C or directly analyzed for nucleotide composition.

Ribonucleotides were separated by ion-exchange HPLC (Spectra Physics 8000B liquid chromatograph equipped with a Partisil Sax 10 column with dual wavelength detection at 254 and 280 nm). Elution was performed at 40°C, at a flow rate of 1.3 ml/min, with a ternair system containing two phosphate buffers (0.05 M KH_2PO_4 pH 3.35 and 0.25 M KH_2PO_4, 0.5 M KCl, pH 5.25) and water. All components contained 2% acetonitrile. Details of the gradient profile are given by De Abreu et al. (1982).

The energy charge (EC) was calculated from the adenine nucleotide content by the standard formula:

$$EC = \frac{ATP + 0.5\ ADP}{ATP + ADP + AMP}$$

5.2.2.5. Retinoid analysis.

Extraction and analysis of retinoids was performed as described in chapter 2 (section 2.1.3).

5.3. RESULTS.

5.3.1. EVALUATION OF CONDITIONS SUITABLE FOR SHORT-TERM INCUBATION.

5.3.1.1. Selection of incubation conditions.

In order to be able to study cellular metabolism and its regulation in vitro it is not only vital to isolate intact cells but also to develop an incubation system in which the cells can be kept viable over a certain time-period to maintain the regular cellular physiology. In accordance with the general experience with mammalian cells we opted for incubation at 37°C in a humidified atmosphere of 95% O_2/5% CO_2. For the cell incubation three media were evaluated. (i) RPMI 1640 DM, (ii) Krebs Ringer salt solution and (iii) Perfusion Buffer. RPMI 1640 has been used before in RPE explant/eye cup experiments (Feeney and Mixon, 1976; Essner et al., 1978; Rosenstock et al., 1980; Markowitz et al., 1981; Goldhar et al., 1984; Seyfried-Williams et al., 1984; McKechnie et al., 1986). Krebs-Ringer solution is a very generally employed cell incubation medium. Perfusion buffer was included since this buffer is used during isolation of the RPE cells (section 3.2.2.1 and 3.2.2.2).

5.3.1.2. Evaluation criteria.

To evaluate whether the isolated RPE cells are able to maintain their structural and metabolic integrity in the course of the in vitro incubation, we analyzed (i) the retention of cellular retinol binding protein, a small cytoplasmic protein, and (ii) their energy charge. Small cytoplasmic proteins will diffuse into the incubation medium when cells develop membrane defects. The energy charge and nucleotide content is a good indicator for cellular viability, since cells in bad condition are not able to maintain a steady state ATP level.

-i- Structural integrity.

The integrity of the plasmalemma, an established parameter of structural integrity of a cell, is generally evaluated by means of viability dyes or by the retention of cytoplasmic proteins like lactate dehydrogenase (LDH M_r 135 kD). However, the RPE cell

contains a high concentration of the cellular retinol binding protein, cRBP (Saari et al., 1984; appendix). Since cRBP is a rather small protein (M_r 15 kD), it was considered a better candidate to examine the integrity of the RPE plasmalemma during prolonged incubation than LDH.

Fig. 5.1 shows the cRBP content of cells incubated in either RPMI 1640 DM or perfusion buffer. The intracellular cRBP concentration maintained a steady state level during at least 8 hr of incubation. This indicates that in both media RPE cells are able to maintain an intact permeability barrier for all protein components. Incubation in bicarbonate buffered Krebs Ringer caused serious problems since the gassing by carbogene, required to maintain the pH, seriously deteriorated the cells. However, upon replacement of bicarbonate by HEPES, complete retention of cRBP was observed as well (not shown).

-ii- Metabolic integrity.

Retention of cRBP, allthough an important parameter for structural integrity and vitamin A metabolism, does not provide an unequivocal criterium for metabolic integrity of the cell. For this

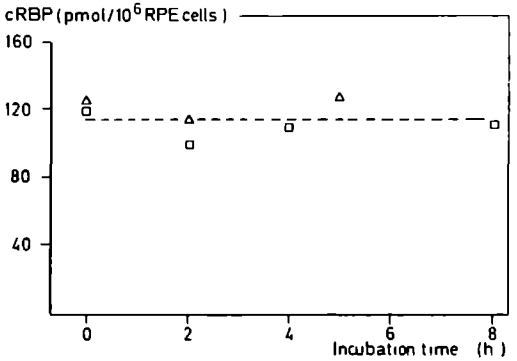


Fig. 5.1
Structural integrity of RPE cells during incubation in perfusion buffer (triangles) or RPMI 1640 DM (squares).
The structural integrity was evaluated by the retention of cellular retinol binding protein cellular retinol binding protein, during incubation. Hereto, the amount of cRBP was determined on cells obtained after incresing incubation times and was expressed in pmol/10^6 cells.

aspect, we considered the energy charge (EC) a much more reliable indicator. The energy charge relates the amount of high energy ATP equivalents to the total adenine nucleotide content and is calculated as:

$$EC = \frac{ATP + 0.5\ ADP}{ATP + ADP + AMP}$$

In a viable cell where catabolic and anabolic processes are in dynamic equilibrium, the energy charge is high and usually in the range of 0.8 - 0.95 (Stryer, 1975).

The adenine ribonucleotide content of RPE cells incubated in the three media is shown in table 5.1. During incubation in perfusion buffer the retinal pigment epithelium cells exhibited an alarming drop in the energy charge and a precipitous decrease in the total adenine nucleotide content. Upon incubation in Krebs Ringer, a slow increase in the energy charge was detected to still suboptimal levels but the total adenine nucleotide pool actually decreased. However, upon incubation in RPMI 1640 DM a steady increase of both the energy charge and total adenine nucleotide level was observed which leveled off to a healthy level between 2 and 4 hr of

Table 5.1. EVALUATION OF THE METABOLIC INTEGRITY OF ISOLATED RPE CELLS DURING SHORT-TERM INCUBATION.

		METABOLIC PARAMETER		
INCUBATION TIME	INCUBATION MEDIUM	ATP*	TOTAL A*	ENERGY CHARGE
	Perfusion buffer	390	540	0.79
T=0 hr	Krebs Ringer	370	370	0.58
	RPMI 1640 DM (4)	320 ± 37	600 ± 50	0.69 ± 0.02
	Perfusion buffer	-	-	-
T=2 hr	Krebs Ringer	-	-	-
	RPMI 1640 DM	390	720	0.73
	Perfusion buffer	30	90	0.47
T=4 hr	Krebs Ringer	260	490	0.66
	RPMI 1640 DM (3)	500 ± 143	730 ± 260	0.82 ± 0.01
	Perfusion buffer	-	-	-
T=8 hr	Krebs Ringer	-	-	-
	RPMI 1640 DM (2)	650 ± 90	900 ± 160	0.82 ± 0.01

*: ATP and total A (=total adenine nucleotide pool) in pmol/10^6 RPE cells. Number of determinations are given between parentheses.

incubation. The cells maintained their energized state for at least 8 hours. No ATP or ADP was detected in the incubation medium throughout the entire icubation.

In perfusion buffer the metabolic condition of the cells rapidly deteriorated, while in Krebs Ringer they could not really recover from the isolation shock. Since, RPMI 1640 DM allowed RPE cells to recover and maintained 'good health' even after 8 hr of incubation, this medium was used in further investigations.

5.3.2. RETINOID STATUS DURING SHORT-TERM INCUBATION OF RPE CELLS.

The retinoid status of the RPE cells was investigated in as far as it is relevant to the visual cycle: content as well as isomeric composition of retinol, retinaldehyde, retinylester (see fig. 1.6) and the effect of the dark/light history.

For convenience the last steps in the RPE cell isolation, detachment of the cells from Bruchs membrane and harvesting of the cells, were, for convenience, carried out under fluorescent light (normal lab illumination). A comparison with the retinoid status of cells isolated completely in the dark revealed no significant differences.

The retinoid status of RPE cells, incubated in RPMI 1640 DM, was monitored over a period of 6 hr (fig. 5.2). The large majority of the cellular retinoid is presented as the retinyl ester (ca 90%), while the retinol and the retinaldehyde fraction both contributed about 5 %. During incubation a slight but not significant decrease in total retinoid content was apparent (ca 10% over 6 hours). The larger part of this decrease (ca 75%) originated in the retinylester fraction , the remainder was due to a decrease in retinol content. The retinaldehyde fraction remained constant. The decrease in retinol content is relatively large: over 50% after 6 hr incubation. The decrease in cellular retinoid content was not caused by leakage out of the cell or secretion since the 'missing' retinoids were not recovered in the incubation medium. The data suggest that during incubation metabolic conversion of retinol occurred into components which were not identified by our HPLC set-up.

The total retinoid status of the RPE cells did not depend on whether incubation was performed in the light or in darkness.

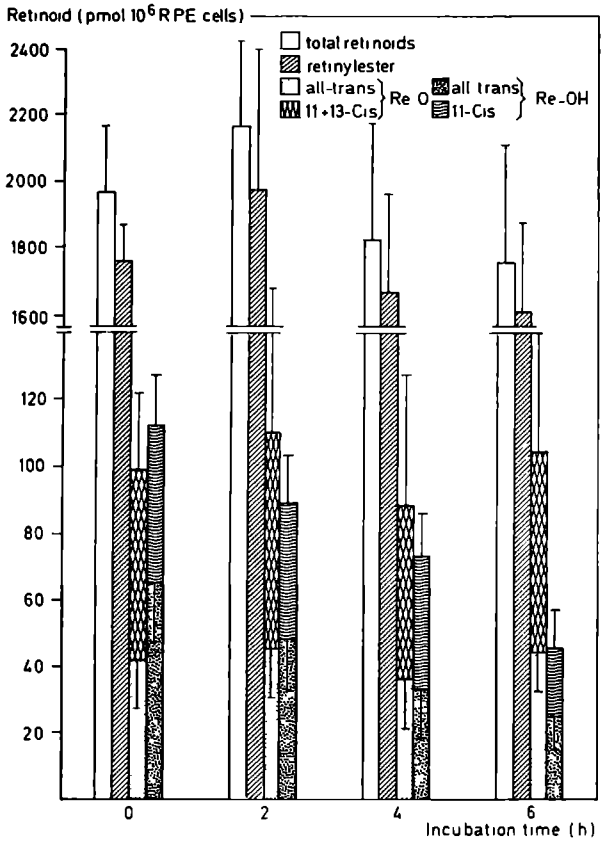


Fig. 5.2
Retinoid status of the RPE cells during incubation in RPMI 1640 DM. Retinoid content is given in pmol/10^6 RPE cells. Retinol (Re-OH) and retinaldehyde (Re=O) are subdivided according to isomeric composition. The speckled part represents the all-trans configuration, the hatched part represents 11-cis retinol and the diamond part represents 11-cis + 13-cis retinaldehyde. Error bars represent S.D. of 4 determinations.

Incubation in the light however affected the isomeric composition of the ester population (fig 5.3), apparent as a slow conversion of the 11-cis fraction into the all-trans. No effects could be detected on the isomeric state of retinol or, rather surprisingly, retinaldehyde. Of the retinoids the latter usually appears to be the most sensitive to photoisomerization in vitro.

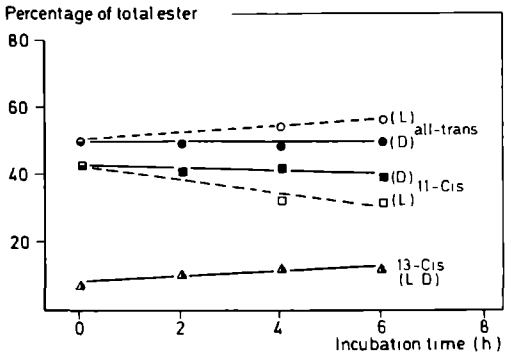


Fig. 5.3
Isomeric composition of retinylester fraction in isolated RPE cells during incubation in light or darkness.
The retinylester fraction was collected and subsequently saponified to the corresponding retinols, which were analyzed for the isomeric composition by HPLC (see chapter 2). The isomers are expressed as percentage of total retinylester. The oper symbols (circles, squares and triangles) represent incubation ir the light, the filled ones represent incubation in the darkness.

5.4. DISCUSSION.

5.4.1. ISOLATED RETINAL PIGMENT EPITHELIUM CELLS CAN BE USED FOR SHORT-TERM INCUBATION STUDIES.

Detailed analysis of the metabolic routes in RPE cells, like those functional in the visual cycle, requires the study of RPE cells in vitro. In cell culture RPE cells underwent serious *dedifferentiation, as demonstrated by a dramatic loss of vitamin* A handling capacity (loss of the retinoid binding proteins and decreased vitamin A uptake and esterification, cf chapter 4). Hence, it was obligatory to investigate, whether, as an alternative, an incubation system for short-term metabolic studies could be developed. For this purpose, isolated RPE cells were incubated under various conditions and their performance scored by two independent parameters: energy charge and adenine nucleotide content as an indicator for the metabolic integrity and retention of a small cytoplasmic protein cellular retinol binding protein, as an indicator for the structural integrity.

5.4.1.1. Metabolic integrity.

The ability of a cell to perform its regular cellular functions depends very much on its energetic potential. This parameter is usually expressed as the energy charge of the cell, which gives the relative amount of high energy equivalents. In healthy tissue, when anabolism and catabolism are in dynamic equilibrium, this parameter lies in the range of 0.8 - 0.95. The capacity of a cell to keep up its energy store (total adenine nucleotide) is another indicator for the metabolic potential of the cell. Together we consider these parameters as good evaluators for the 'metabolic integrity' of the cell.

The metabolic integrity of isolated RPE cells appears to be very well conserved during incubation in RPMI 1640 DM. The energy charge rises to a healthy steady level of 0.82 $\pm$ 0.01 (5). Likewise, the total adenine nucleotide content increases during incubation to a level of 800 $\pm$ 200 pmol/10^6 cells. A similar behavior was observed for the guanine nucleotides (energy charge: 0.77 $\pm$ 0.06 (5); total: 195 $\pm$ 45 (5)). Data on these parameters in bovine eye tissue are lacking in the literature, but a global comparison can be made with figures based on the analysis of frog tissue. From the data of Barbehenn et al. (1985) we derive an energy charge of 0.9 and a total adenine nucleotide content of 7.1 mmol/kg dry weight for frog RPE. Assuming a dry weight for bovine RPE of about 200 ug/10^6 cells (Berman and Feeney, 1976; Saari et al., 1977; Berman, 1979), we arrive at a total adenine nucleotide content after 8 hours of incubation of 4 - 5 mmol/kg dry weight, which is in the same range as calculated for frog tissue.

5.4.1.2. Structural integrity.

The structural integrity as evaluated by the retention of cellular retinol binding protein proved not to be very discriminative. Complete retention was observed under all three incubation conditions even where a dramatic decrease in metabolic integrity was apparent. Hence, retention of macromolecular intracellular components is not a reliable indicator for overall cell condition. Loss of structural integrity probably only occurs in more advanced stages of deterioration.

5.4.1.3. Conclusion.

Incubation of isolated RPE cells in RPMI 1640 DM allows complete retention of cRBP, an essential protein in the retinoid metabolism, and a nearly constant overall retinoid status. Further it allows recovery of the energetic state to a level, which is maintained up to at least 8 hr of incubation. This token of good health gives us confidence that the developed incubation system is suitable for short-term investigation of the metabolic activities of RPE cells.

5.4.2. RETINOID COMPOSITION OF FRESHLY ISOLATED BOVINE RPE CELLS.

5.4.2.1. Retinoid composition.

Table 5.2 compiles available literature data on retinoid composition of bovine RPE cells. The values show little agreement on total retinoid content: 1640 - 7500 pmol/10^6 RPE cells, but they do agree with respect to retinylester being the predominant fraction. The reason for this discrepancy is presently unclear: light/dark adaptation of the eyes, the nutritional status of the donor animal, possible seasonal effects, and isolation artefacts

Table 5.2 RETINOIDS IN BOVINE RETINAL PIGMENT EPITHELIUM.

RETINOIDS[1] (% 11-cis)[2]				
RETINYLESTER	RETINALDEHYDE	RETINOL	TOTAL	REF
2240	-	200	2440	[A]
2660	640	300	3600*	[B]
1060	500	80	1640@	[B]
7500 (50%)	64 (85%)	40 (3%)	7500	[C]
1760±120 (40%)	100±50 (55%)	110±30 (45%)	1970±150@	[D][3]

1: Amounts are given in pmol/10^6 cells.
2: The percentage 11-cis retinoids is given between parentheses.
3: data from 9 different RPE cell isolations (mean ± S.D.).
*: eyes were light adapted.
@: eyes were dark adapted.
A: Berman et al., 1979; B: Adler and Martin, 1982; C: Bridges et al., 1984
D: This paper.

may all affect the retinoid status.

Only Bridges et al. (1984) present data on the isomeric composition of the retinoids in bovine RPE. Again there is little agreement with our findings. Remarkably, these two data sets agree rather well with respect to the average content of 11-cis retinaldehyde (54 vs 55 pmol/10^6 RPE cells) and all-trans retinol (39 vs 60 pmol/10^6 RPE cells).

5.4.2.2. Retinoid binding proteins in relation to retinoid composition.

We measure a cellular retinol binding protein content of the isolated bovine RPE cells of 120 $\pm$ 15 pmol/10^6 cells, which comes to about 600 pmol/eye. This agrees well with the figure of about 500 pmol/eye reported by Saari et al. (1984) based on purification studies. Assuming that all-trans retinol is bound to cRBP, we conclude that only about 50% of the cRBP is occupied. Although similar observations have been reported for other tissues (Chytil and Ong, 1979), this conclusion disagrees with data reported by Saari et al. (1982), which suggest complete saturation of cRBP with all-trans retinol in RPE.

A similar situation exists for the 11-cis retinoid binding protein cRA1BP. This protein is present in an amount of about 140 pmol/10^6 RPE cells (Saari et al., 1984). Again it was reported to be fully saturated with the endogenous ligand 11-cis retinaldehyde (Saari et al., 1982). However, a 11-cis retinaldehyde content of ca 55 pmol/10^6 RPE cells would only give approximately 40% saturation, rising to about 70% when 11-cis retinol is taken into account.

In a cell with high periodic influx of retinol, a high metabolic flexibility seems logical and only partial coverage of retinoid binding proteins under conditions of low retinol influx might be expected. Nevertheless, the discrepancies mentioned above clearly require further investigation. Important parameters like light/dark history of the donor animals, cell damage during isolation and extraction efficiency of retinaldehyde (Groenendijk et al., 1980) should then be very well controlled.

5.4.3. RETINOID FLUXES DURING CELL INCUBATION.

The overall picture which emerges from the incubation studies is that only small net retinoid fluxes or shifts occur from one pool to another. Whether this reflects a static or dynamic 'equilibrium' cannot be inferred from the present study, where absolute levels have been determined. Only the retinol level shows a significant decrease (ca 50% after 6 hr of incubation). This is not due to leakage out of the cells. Use of retinol for other metabolic purpose is a likely cause of this decrease, since retinoids are known to affect genetic expression and synthesis of glycoproteins. The slight decrease in retinylester might reflect partial metabolic compensation by hydrolysis of retinylester for such utilization. This aspect again deserves further investigation.

Remarkably, incubation in the light appeared to affect the isomeric composition of the retinylester pool, while usually retinaldehyde, even when bound to the binding protein cRAlBP, is much more prone to undergo light induced isomerization. This phenomenon might be explained by assuming a dynamic equilibrium between the 11-cis retinoid pools: the 11-cis retinaldehyde level would then be maintained at the expense of the 11-cis retinyl ester pool. The correlation with the decrease in 11-cis retinylester content in frog RPE during light adaptation (Bridges, 1976) is noteworthy. Also this aspect deserves further investigation.

5.4.4. CONCLUSION.

The presented results demonstrate that isolated bovine RPE cells can be maintained in in-vitro incubation for at least 8 hr. This system allows short-term metabolic studies for a range of cellular functions. The high stable, endogenous retinoid content however renders the analysis of metabolic routes relevant to the visual cycle rather complex when only endogenous retinoids can be analyzed. A better approach would be to present labeled retinoids to the cells and to follow label-distribution over the various retinoid fractions in time. Such studies will be described in the following chapter.

Chapter 6

METABOLISM OF ALL-TRANS RETINOL IN ISOLATED BOVINE RETINAL PIGMENT EPITHELIUM CELLS.

6.1. INTRODUCTION.

The visual cycle, i.e. the recycling of visual pigment chromophore involving photoreceptor cell and retinal pigment epithelium (summarized in fig 1.7) has a relatively long history of fragmentary elucidation. The present status, recapitulated in fig 6.1, still presents a mixture of facts and speculations. Bleaching of rhodopsin and subsequent reduction and migration of the liberated chromophore, finally resulting in esterification of retinol in the RPE cells, are well documented (cf section 1.4.3.1). Several open spots persist, however, in the reverse route effectuating regeneration of rhodopsin.

Regeneration of rhodopsin requires the conversion of retinylester eventually into 11-cis retinaldehyde and delivery to the outer segments. In this process three distinct intermediate steps can be distinguished: (i) hydrolysis of the retinylester to retinol which has to be (ii) isomerized and (iii) oxidized to form 11-cis retinaldehyde.

Several major questions still remain unanswered like 'which retinoid-species is substrate for isomerization and which one for oxidation' and 'which species is the transport form and what route does it take'. It is generally accepted that the all-trans retinyl-ester is a poor substrate for the isomerization activity. This

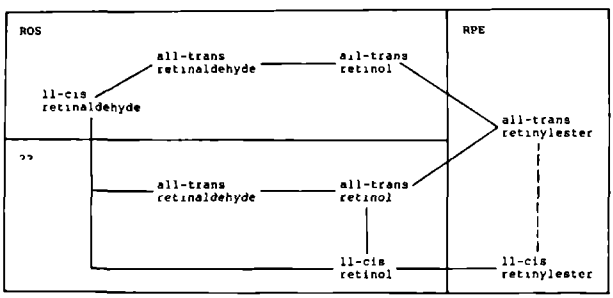


Fig 6.1.
Present status of facts and speculations in the visual cycle.
Established retinoid conversions in the visual cycle which occur in the rod outer segments or retinal pigment epithelium are shown in the boxes marked ROS and RPE. Expected transitions, of which localization or transition pathway still are a matter of conjecture, are displayed in the box marked ??.

leaves two alternative modes: (i) all-trans retinol is isomerized into 11-cis retinol with subsequent oxidation into 11-cis retinaldehyde. (ii) All-trans retinol is oxidized into all-trans retinaldehyde followed by isomerization into 11-cis retinaldehyde. The first mode is postulated in the most current model for the visual cycle (fig 1.8). This model proposes that in view of the distribution of 11-cis retinoid binding protein, cRAlBP, and its endogenous ligand (table 1.2), isomerization takes place in the Müller cell and oxidation in the RPE cell.

Since the RPE cell is an essential element in the visual cycle (see also chapter 5), the present knowledge of the retinoid metabolism in the RPE cell, relevant to the regeneration process, will be discussed first.

6.1.1. ESTERIFICATION AND ESTER HYDROLYSIS.

The retinylester in RPE cells is considered only to function as a retinoid storage form in analogy to the situation in liver. A flexible 'storage form' requires the presence of enzymes capable of rapidly interconverting this form with the 'metabolic form'.

Retinol specific esterifying activity has been demonstrated in the microsomal fraction of the RPE cell (Krinsky, 1958; Berman et al., 1980; Fong et al., 1983). Interestingly, all-trans retinol bound to cellular retinol binding protein, cRBP, is a better substrate for this enzymatic activity than free retinol (apparent Km of 5.5×10^{-6}M vs 16.6×10^{-6}M; Berman et al., 1980). This derivatization is not restricted to the all-trans configuration since 11-cis retinol complexed with cellular 11-cis retinoid binding protein, cRAlBP, is esterified at an even higher rate than cRBP bound all-trans retinol (Saari et al., 1984). It has not been established whether the same enzyme is involved in both reactions.

Only very recently, the presence in the RPE of a retinylester hydrolase, catalyzing the reverse reaction, has been reported (Berman et al., 1985; Blaner et al., 1985b; Ishizaki et al., 1986; Tsin and Lam, 1986).

These findings demonstrate that both esterification to a transient store and retinylester hydrolysis, the return-path to regeneration, can be carried out in the RPE.

6.1.2. OXIDATION AND REDUCTION.

It has previously been demonstrated in our laboratory that a stereospecific 11-cis oxidoreductase is present in bovine RPE (Lion et al., 1975). 11-Cis retinol and 11-cis retinaldehyde bound to cellular 11-cis retinoid binding protein have been shown to be potential substrates (Saari et al., 1984). The very presence of this enzymatic activity, does not answer the question which process occurs first in the regeneration pathway: isomerization or oxidation of retinol. In view of the observation that in the RPE the endogenous ligand of the 11-cis retinoid binding protein, cRALBP, is almost exclusively 11-cis retinaldehyde (Saari et al., 1982), the current model for the visual cycle (fig 1.8) proposes that the main function of the 11-cis retinol oxidoreductase is to convert 11-cis retinol into 11-cis retinaldehyde.

In addition, an all-trans oxidoreductase, active on all-trans retinol and retinaldehyde, is present in the RPE as well (Zimmerman et al., 1975). Its total activity is about 4-5 times lower than that of the 11-cis oxidoreductase activity (Lion et al., 1975; Zimmerman et al., 1975). It cannot be excluded that both enzymatic activities represent one enzyme, which has the 11-cis isomer as the preferred substrate.

These findings demonstrate that an essential step in the rhodopsin regeneration pathway, i.e. interconversion of retinol and retinaldehyde, irrespective of geometric configuration, can be carried out in the RPE cell as well.

6.1.3. ISOMERIZATION.

The most essential step in the regeneration pathway, isomerization of the all-trans into the 11-cis configuration, has remained the most obscure. First of all, it is still not clear whether isomerization occurs at the level of the alcohol or the aldehyde. Further, no consensus has yet been achieved, whether or not an enzyme is involved. Photoisomerization, an important mechanism for visual pigment regeneration in invertebrates (Schwemer et al., 1971; Hara et al., 1981a), does probably not occur in vertebrates, since the regeneration pathway is completely active in darkness (dark adaptation).

In vitro, isomerization of all-trans retinaldehyde in darkness can be strongly catalyzed by small amino group containing compounds (e.g. phosphatidylethanolamine, abundantly present in vivo; Groenendijk et al., 1980b; Bernstein et al., 1986a,b). This however leads to a mixture of all-trans and cis isomers, and produces the 11-cis isomer only very inefficiently (Groenendijk et al., 1980b). Since, under normal conditions, in vivo only the all-trans and 11-cis form are detected, the general opinion favors enzymatic catalysis, (however see Bernstein et al., 1985). Further, many authors believe that the isomerization takes place at the retinaldehyde level, since this retinoid shows the by far highest rates of isomerization in vitro (Hubbard, 1956; Rotmans et al., 1972; Futterman and Rollins, 1973; Groenendijk et al., 1980b; Bernstein et al., 1986a,b). However, evidence supporting isomerization at the retinol level, as postulated in the current model, has recently been presented (Bernstein and Rando, 1986).

Finally, the exact site of isomerization is a matter of conjecture as well. No solid evidence whatsoever is even available which allows to locate the isomerization activity in either RPE or retina.

6.1.4. EXPERIMENTAL APPROACH.

Evidently, several enzymatic activities relevant for the visual cycle, have been demonstrated in the RPE. Nevertheless the question whether the RPE is involved in functions other than the 'overflow' and 'back up' storage of retinol has still not been answered. In order to address this question, a detailed analysis of the retinoid pathways in the intact RPE cell needs to be performed. As yet, the retinoid metabolism in the RPE cell has only been investigated in cell homogenates or on mechanically isolated RPE cells. The latter approach has been shown to seriously affect the cell integrity (Saari et al., 1977). The new isolation technique we have developed for bovine RPE cells produces viable cells in high yield (Timmers et al., 1984; chapter 3), which can be maintained under appropriate short-term incubation conditions for metabolic studies (chapter 5). This system is potentially very suitable for studies on the retinoid processing in the RPE cell. Our approach was to mimick the first step of the visual cycle (influx of retinol into RPE

following rhodopsin bleaching) by presenting labeled all-trans retinol to the isolated cells and follow label incorporation into other isomers or retinoids in time.

This chapter describes the incubation of isolated bovine RPE cells with ^{3}H all-trans retinol, incorporated into phosphatidylcholine carrier vesicles. The label distribution over the retinoid isomers and classes present in the RPE cells is followed during two hours of incubation in the dark. The potential of this system for this type of investigation and the implications for current models of the visual cycle will be discussed.

6.2. MATERIALS AND METHODS.

6.2.1. MATERIALS.

Unlabeled all-trans retinol was obtained from Eastman Kodak Company (Rochester, New York, USA), labeled (11-12-di-^{3}H-) all-trans retinol (specific activity: 55 Ci/mmol) from Amersham (Amersham, UK), soya lecithin from Lipid Products (South Nuthfield, Surrey, UK) and Aqua Luma Plus scintillation fluid from Lumac:3M BV (Schaesberg, The Netherlands).

6.2.2. METHODS.

6.2.2.1. Preparation of phosphatidylcholine carrier vesicles.

450 µl of soya lecithin solution (10 mg phospholipid/ml chloroform:methanol (75:25)) was dried in a stream of nitrogen followed by exposure to 30 min of high vacuum. To the phosphatidylcholine residue, 600 µl of RPMI 1640 DM was added. Liposomes were prepared by vigorous shaking for 10 min and transformed into small vesicles by sonication on ice (Branson B12 sonifier with microtip) until an opalescent dispersion was achieved (10 - 15 min). The vesicles were prepared one day before use and kept at $4^{o}C$ under nitrogen.

6.2.2.2. Purification of ^{3}H all-trans retinol and incorporation in soya phosphatidylcholine carrier vesicles.

All manipulations were carried out under dim red light in order to prevent photo-isomerization of all-trans retinol. 75 µl

^{3}H-labeled retinol (75 µCi) was mixed with the desired amount of unlabeled retinol in ethanol. The solvent was evaporated in a stream of nitrogen and the residue dissolved in hexane/di-ethylether (50:50; v/v). Subsequently, all-trans retinol was purified from contaminating 13-cis retinol by semi-preparative high pressure liquid chromatography (LiChrosorb Si 60-5; 250 x 9 mm; flow rate 3 ml/min; eluent:hexane/di-ethylether (50:50); detection at 328 nm). Baseline separation of all-trans retinol and 13-cis retinol was achieved. The all-trans peak was collected, the solvent evaporated by a stream of nitrogen and the residue was dissolved in 5 µl ethanol. To this solution 400 µl of a dipersion of phosphatidylcholine carrier vesicles was added. Under careful but thoroughly mixing under nitrogen the all-trans retinol incorporated into the vesicle membranes within several minutes. Of the resulting ^{3}H all-trans retinol carrier phosphatidylcholine dispersion, 30 ul aliquots were administered to 800 µl preincubated RPE cell suspension or control incubations.

6.2.2.3. Cell isolation.

Isolation of bovine RPE cells was performed via the perfusion technique as described in chapter 3 (section 3.2.2.1 and 3.2.2.2).

6.2.2.4. Determination of cell density.

Cell density was determined by means of a DNA assay carried out as described in chapter 2 (section 2.2.2).

6.2.2.5. Cell incubation.

Short-term incubations of the isolated RPE cells was carried out as described in chapter 5 (section 5.2.2.3).

Prior to administration of ^{3}H-labeled all-trans retinol in phosphatidylcholine vesicles, RPE cells were preincubated for 2 h to recover from the isolation shock and to restore their energy housekeeping.

After various time intervals, incubation mixtures were harvested and RPE cells were sedimented (900 x g; 3 min; 4^{o}C). 500 µl of the supernatant was set aside for analysis of the incubation medium. Incubation wells and cellpellets were washed with 1 ml icecold RPMI 1640 DM, and the final cellpellet was resuspended in 500 µl icecold RPMI 1640 DM. Aliquots of 50 µl were taken of the

cell suspension and incubation medium to determine ^{3}H label recovery. Cell suspensions and incubation medium were then frozen rapidly and stored at -20^{o}C for retinoid analysis.

6.2.2.6. Retinoid analysis.

Extraction of retinoids was carried out as described in chapter 2 (section 2.1.3).

Retinoids were separated by HPLC by gradient elution to ensure complete separation of the retinaldehyde from the retinylester fraction. The isomeric status of the retinylester and aldehyde fractions were determined as the corresponding retinols after saponification and reduction, respectively (chapter 2; sections: 2.1.4.1, 2.1.4.2 and 2.1.5).

6.2.2.7. Liquid scintillation analysis.

Samples of cells, of incubation medium and of HPLC eluate fractions of 20 sec were collected in 6 ml scintillation vials. Four ml scintillation fluid (Aqua Luma) was added and after thoroughly mixing the ^{3}H label was counted with a liquid scintillation analyzer (United Technologies Packard Minaxi TriCarb 4000; 5 min counting time).

6.3. RESULTS AND DISCUSSION.

6.3.1. EXPERIMENTAL SET-UP AND CONTROL EXPERIMENTS.

6.3.1.1. Label carrier.

Since retinol has a very low solubility in aqueous media and under such conditons will aggregate and adhere to whatever surfaces present (see appendix), administration of retinol to the RPE cells requires a suitable stable carrier. While the interphotoreceptor retinoid binding protein, IRBP, which probably functions as the retinol transport vehicle in the interphotoreceptor matrix between photoreceptor and retinal pigment epithelium, seems a logical choice for a carrier, this approach could not be brought under sufficient experimental control. Reproducible loading of IRBP with retinol could not be achieved and the system was not stable in time. Further, this system, in our hands, produced undesirable high levels of 'aspecific' isomerization (15% 13-cis after 30 to 60 min).

Since IRBP has a relatively low substrate specificity and a low affinity for retinol (Kd ca 10^{-6}M), it actually behaves as a non-specific carrier. Currently, there are no indications for IRBP binding sites on RPE cells (Hollyfield et al., 1980b). Hence, it occurred to us that it might be replacable by another aselective carrier system, phospholipid vesicles, which have demonstrated their potential before. Vesicles of phosphatidylcholine have proven to be reliable and stable retinol carriers, with a high transfer rate and large capacity (Rando and Bangerter, 1982; Groenendijk et al., 1984). Even the geometric configuration of the retinoids appeared to be very stable in this system and they were succesfully used in regeneration experiments (Yoshikami and Noll, 1978). In addition, these vesicles are very easily prepared and loaded with retinol (Rando and Bangerter, 1982; Groenendijk et al., 1984).

The isomeric configuration of all-trans retinol incorporated into phosphatidylcholine carrier vesicles appeared to be sufficiently stable under the developed cell-incubation conditions (chapter 5) to allow incubation times of at least 2 hr. In that interval the 13-cis content slowly and continuously increased from less than 2% at t=0 to maximally 4%. Isomers other than all-trans and 13-cis were not detected. It should be noted however, that the presence of cellular membranes might somewhat accelerate the rate of such aspecific isomerization reactions (Groenendijk et al., 1980b).

6.3.1.2. Cell incubations.

In order to determine whether incubation with phosphatidylcholine vesicles would influence the steady state of the endogenous retinoids, control incubations were carried out containing RPE cells with un-loaded phosphatidylcholine carrier vesicles. Both cells and incubation medium were analyzed for retinoids at various time intervals. These special incubation conditions appeared not to have significant effects on the retinoid status, which was comparable to that measured in cells incubated without vesicles (chapter 5). In the incubation medium a constant amount of retinylester (107 $\pm$ 10 pmol/10^6 RPE cells; ca 5% of the total) and all-trans retinol (16 $\pm$ 5 pmol/10^6 RPE cells) was found. This is probably due to RPE cells damaged during isolation and other initial incubation trauma. In agreement with earlier observations

(chapter 5) the total cellular retinoid content slightly decreased in time, however now predominantly by loss of 11-cis retinylester.

During incubation of RPE cells with labeled all-trans retinol, no labeled retinol-metabolites were detected in the incubation medium indicating that retinoid transfer is vectorial (only vesicle to cell).

6.3.1.3. Label supply and uptake.

Dark adapted bovine eyes contain approximately 50 nmoles of rhodopsin (de Grip et al., 1980) and 5 x 10^6 RPE cells. Dependent on the illumination levels, rhodopsin bleaching will effectuate a retinol flux into the RPE up to 5 nmol/10^6 RPE cells · min. Care had been taken that the retinol extraneously presented to the incubated cells did not exceed this range and amounts varying between 1.1 and 5.8 nmol/10^6 RPE cells were used. However, these values cannot be directly compared to in vivo flux densities, since the ratio of aqueous volume to cell membrane surface is at least 10-fold larger for the cell suspension than in vivo. Hence, the actual flux density in our experimental conditions compares with relatively low illumination levels in vivo.

Incubation of retinol-loaded vesicles with RPE cells resulted in appreciable transfer of label to the RPE cells (fig. 6.2). Interestingly, the relative uptaken of label by the RPE cells was independent of the total retinol-supply. Hence, the collision rate and contact time between vesicles and cells are probably rate-determining. The uptake of retinol by the cells showed a rapid initial phase (ca 1% of the total label/min), which after about 20 min slowed down to a constant rate of about 0.1% label/min. Uptake was mainly due to transfer of retinon from vesicles to cells and not by fusion of vesicles with cells or endocytosis of vesicles. The contribution of the latter process was determined by labeling vesicles with ^{14}C-cholesteryl-oleate, a non-exchangeable probe, instead of ^{3}H retinol and measuring uptake of ^{14}C-label by the RPE cells. In the initial phase, fusion or uptake of entire vesicles accounted for maximally 5% of the total retinol-uptake (fig. 6.2). In the slower phase up to 50% of the uptake might be due to ingestion of entire vesicles.

The influx of ^{3}H all-trans retinol did not cause significant changes in the endogenous retinoid pools. The latter were

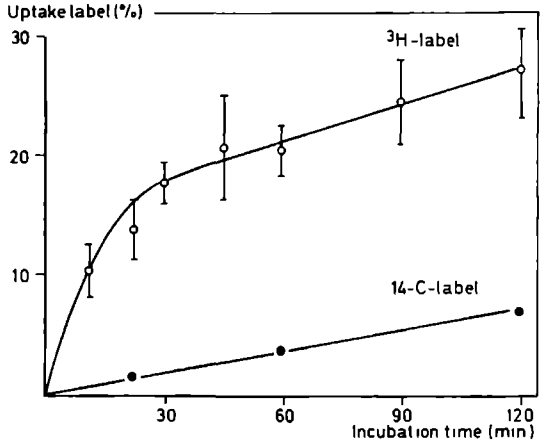


Fig 6.2.
Relative label uptake by retinal pigment epithelium cells from phosphatidylcholine vesicles loaded with either ^{3}H-labeled all-trans retinol or the ^{14}C labeled non-exchangeable probe:cholesteryl-oleate.
At various time points, RPE cells were separated from the incubation medium and the label or ^{14}C label present in the cellpellets was determined by liquid scintillation analysis. After an initial rapid uptake of ^{3}H label, the rate of uptake slows approximately 10-fold. Uptake of the non-exchangeable ^{14}C label is linear in time. The initial retinol uptake maximally contains a 5% contribution by vesicle ingestion. In the slower phase 50% of retinol incorporation might be due to vesicle ingestion.

calculated by subtracting the labeled pool, determined by the label distribution, from the total, determined by spectroscopy. The endogenous pools were not significantly different from control cells, incubated in the absence of ^{3}H-labeled all-trans retinol. Presumably, the pools have a high capacity or a high dynamic range and were not yet saturated under the present experimental conditions. This suggests that eventually the label distribution over the various pools should become similar to the endogenous retinoid, i.e. all pools would then have the same relative specific activity.

The recovery of ^{3}H-label, which scores incubation and manipulation losses, was determined by adding the amount of label recovered the RPE cells to that recovered in the incubation medium, after correction for volume differences, and dividing this sum by the label added at t=0. A constant recovery of 82 $\pm$ 3% was obtained throughout the experiments for all time points.

Evidently the incubated RPE cells exhibit a high capacity for retinol uptake. In the range studied, the extraneous retinol concentration appeared to be the rate-limiting factor in the uptake. The metabolic fate of the retinol, taken up by the RPE cells, was investigated via HPLC analysis of RPE cells incubated during various time intervals.

6.3.2. CELLULAR PROCESSING OF ALL-TRANS RETINOL.

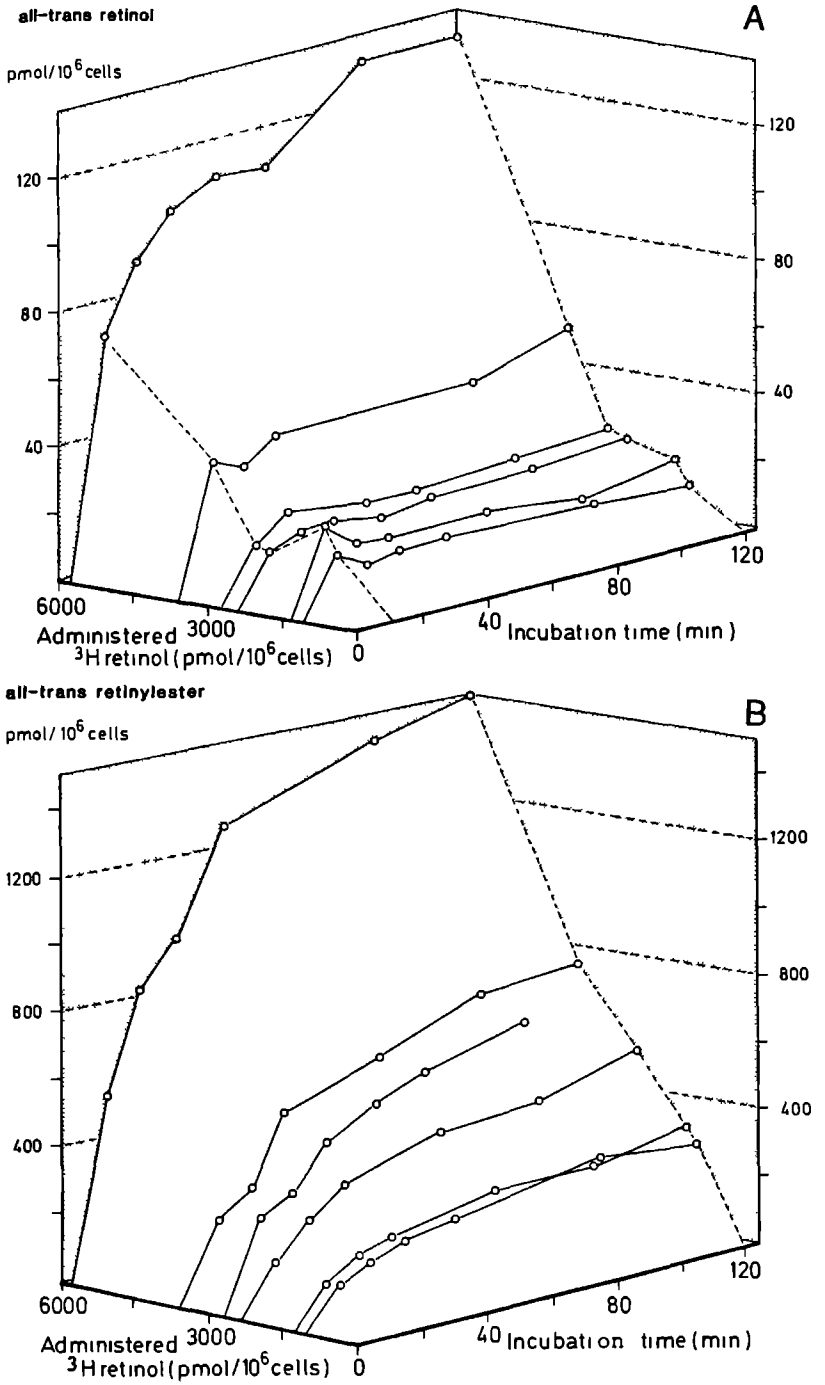
The processing of the internalized retinol was evaluated in dependence of incubation time and administered retinol concentrations. Both the rate of formation and the relative specific activity (the specific activity of the cellular retinoid pool relative to the specific activity of the administered retinol) of the various retinoids, were taken into account. These parameters can give important clues as to the metabolic routes of retinol. First we will discuss the general pattern of the all-trans retinoid and subsequently the 11-cis isomers.

6.3.2.1. All-trans retinol.

The cellular all-trans retinol label demonstrated a rapid initial increase (10 - 20 min) due to uptake (fig. 6.3A) Subsequently, the concentration of labeled all-trans retinol leveled off to a steady state, which represents only 6 to 8% of the total amount of retinol internalized.

Both the initial uptake rate and the steady state level appeared to be strongly, possibly even linearly, dependent of the supplied retinol concentration. This indicates that the administered amount of retinol did not yet exceed the retinol uptake capacity of the RPE cells and that the extracellular amount is still rate-limiting in the cellular processing of retinol. Consequently, the calculated retinoid conversion rates probably represent underestimations. These findings emphasize the enormous capacity of the RPE to internalize and metabolize retinol, probably a prerequisite to handle peak-supplies during high bleaching levels.

It is generally accepted that intracellular all-trans retinol occurs as a complex with cellular retinol binding protein (cRBP). Consequently, the cRBP concentration (120 $\pm$ 15 pmol/10^6 RPE cells)


all-trans retinol
A
pmol/10^6 cells
120
80
40
6000
3000
0
Administered
3H retinol (pmol/10^6 cells)
40
80
120
Incubation time (min)
all-trans retinylester
B
pmol/10^6 cells
1200
800
400
6000
3000
0
Administered
3H retinol (pmol/10^6 cells)
40
80
120
Incubation time (min)

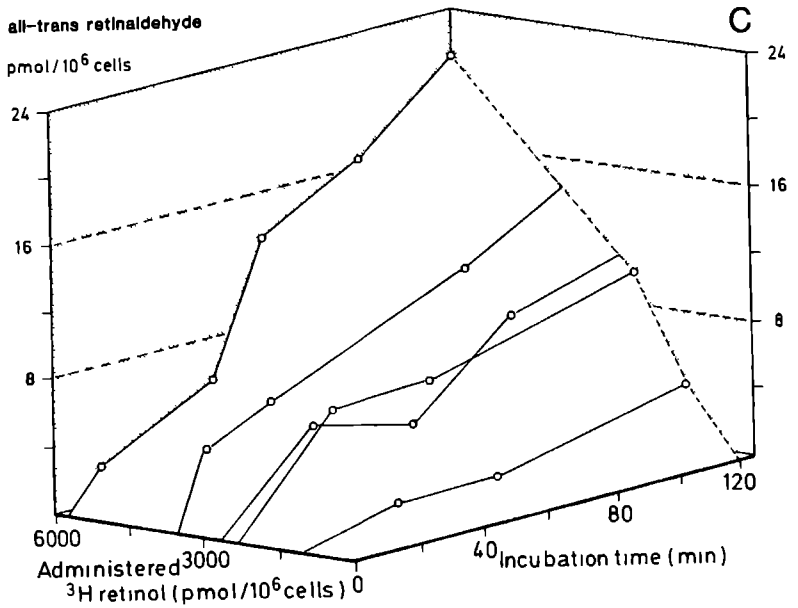


Fig 6.3.
Cellular levels of labeled all-trans retinoids in incubated retinal pigment epithelium cells in dependence of incubation time and labeled all-trans retinol supply.

Cellular all-trans retinoids were separated by means of HPLC and the label content in HPLC eluate fractions of 20 sec was determined by liquid scintillation analysis. A: labeled all-trans retinol; B: labeled all-trans retinylester; C: labeled all-trans retinaldehyde.

Labeled all-trans retinol rapidly levels off and appears to be rapidly further processed. The level of labeled retinylester increases continuously with time and in dependence of the exogenous retinol supply and constitutes the major labeled retinoid in incubated RPE cells. In contrast to retinol and retinylester, all-trans retinaldehyde displays a linearly increase with time.

probably limits the all-trans retinol pool. The cellular retinol concentration (labeled + unlabeled) remained indeed always below the cRBP level, except for the highest retinol concentration administered, where the retinol level (150 pmol all-trans retinol/10^6 cells) slightly exceeded the cRBP level of control cells. The actual cRBP level under the latter conditions was however not measured and it need to be determined whether retinol dependent induction of cRBP levels may occur.

The relative specific activity of the cellular retinol pool gives information on whether the internalized labeled retinol completely mixes with the endogenous retinol pool (ca 40 pmol/10^6 RPE cells). If this were the case, one would expect a rapid rise of the relative specific activity of the total retinol pool to 0.9 - 1 for all extracellular retinol supplies, since the labeled retinol flux is at least 5 to 40 times larger than the endogenous unlabeled retinol content. In contrast, the relative specific activity of the cellular all-trans retinol leveled off after a rapid initial increase, whereby the final level appeared to be strongly dependent on the administered retinol concentration (fig 6.4) and only approached 0.8 at the highest administered retinol concentration. This suggests that the newly internalized retinol is preferentially processed over the endogenous pool and that these pools do not rapidly mix. This also explains the little effect of retinol uptake on the endogenous unlabeled retinol pool size.

6.3.2.2. All-trans retinyl ester.

The all-trans retinyl ester was the by far predominant metabolite of the internalized ^3H all-trans retinol. Its production in time in dependency of the administered retinol concentration is shown in fig. 6.3B. After a rapid initial phase (20 - 30 min), the rate slows down but, unlike the labeled retinol, the retinylester pool showed a continuous increase and no steady state was reached within our incubation time- frame. Hence, the retinylesters are not rapidly further metabolized, which allows to calculate the rate of esterification. It should be kept in mind that these figures are underestimations of the maximal rate, even at the highest retinol supply, since the retinol uptake still seems to be rate limiting under our experimental conditions. At the highest retinol concentration a rate of 54 pmol

retinylester/min · 10^6 RPE cells is calculated. At this rate, a one-shot full rhodopsin bleach (50 nmol) could be processed within 20 min!

At all time points, a linear relation appeared to exist between the retinylester pool size and the administered retinol concentration. This manifested itself also in the relative specific activity of the total ester pool (fig 6.4) which increased both with time and administered retinol concentration. Here, the relative specific activity agrees with the influx of labeled ester into an endogenous ester pool. At the highest administered retinol concentration the ester pool increased in size by 230%! Such a high esterification rate in combination with large dynamic range of the retinylester pool and the low extent of further metabolization under conditions of high retinol intake, fully support the concept that the ester functions as the retinoid storage form.

6.3.2.3. All-trans retinaldehyde.

Labeled all-trans retinaldehyde (fig 6.3C) was only formed in small amounts (< 20% of the labeled all-trans retinol level after 120 min). It should be taken into account that the concentration might be somewhat underestimated due to incomplete extraction (Groenendijk et al., 1980a). Nevertheless, the different samples could be mutually compared since the extraction efficiency was similar for all samples (see chapter 2).

Unlike retinol and retinylester, the labeled retinaldehyde level increased linearly in time. The rate also seemed nearly linearly related to the administered retinol concentration and was maximally 0.15 pmol/min · 10^6 cells. These findings suggests that in metabolic processing of retinol the oxidation route to retinaldehyde is of secondary importance relative to esterification.

The relative specific activity of the labeled retinaldehyde pool (fig 6.4) increased steadily with time and with the administered retinol concentration, suggesting either complete mixing of both the labeled and unlabeled all-trans retinaldehyde pool or only slow further processing of separate pools.

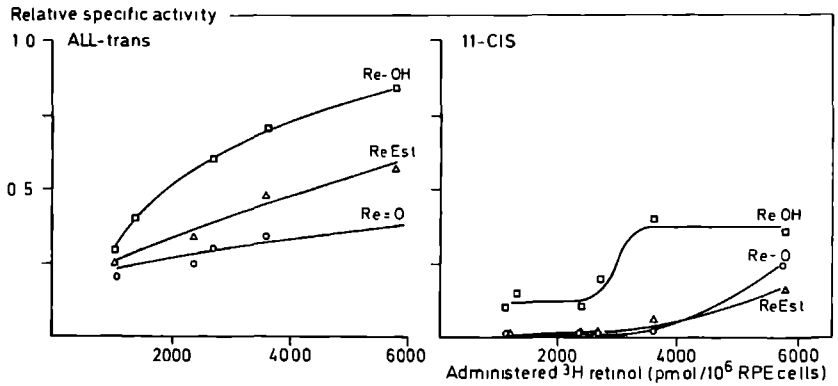


Fig 6.4
Relative specific activity of cellular retinoids in dependence of administered labeled all-trans retinol supply after 120 min incubation.
The relative specific activity (RSA) reflects the ratio of the specific activity of cellular retinoids to the original specific activity of the extracellularly added all-trans retinol. The RSA of the all-trans retinoids show a fairly linear relation with the to extracellular retinol concentration. Despite an uptake of labeled all-trans retinol of 5 to 40 times the endogenous unlabeled all-trans retinol pool, its relative specific activity does not rise above 0.8, which implies preferentially processing of freshly taken up retinol.
The relative specific activity of 11-cis retinaldehyde and 11-cis retinylester only starts to rise after the relative specific activity of 11-cis retinol has increased to a steady level. This strongly suggests that isomerization takes place at the level of retinol. Further, the rise in the RSA of the retinylester seems to precede that of the aldehyde, suggesting the esterification is switched on before oxidation.

6.3.3. ISOMERIZATION.

In addition to the retinol esterification and oxidation activities expected in view of the enzymatic activities, already described for homogenates or subcellular fractions of RPE cells, stereoisomerization was observed as well in every experiment. These findings are the first to demonstrate that geometric transitions of all-trans to 11-cis in the dark can be carried out in the RPE cell!

In vivo, only the all-trans and 11-cis configurations are detected. In these in vitro experiments however, in addition to the all-trans, both the 11-cis and 13-cis configurations were identified in approximately similar amounts. The following arguments are presented that 13-cis formation probably reflects 'non-specific' isomerization: (i) formation of 13-cis isomers, but not 11-cis isomers, is easily catalyzed by amino compounds

(Groenendijk et al., 1980b; Bernstein et al., 1986a,b). (ii) the formation of 13-cis isomers was also detected in time dependent amounts in control incubations (retinol loaded phosphatidylcholine carrier vesicles in the absence of RPE cells). Under these conditions, no trace of 11-cis isomers could be detected! (iii) during manipulation of the retinoids (extraction and HPLC analysis), additional formation of 11-cis isomers was <u>never</u> observed. On the contrary, loss of the 11-cis configuration due to non-specific isomerization is a more likely phenomenon since the 11-cis isomer is energetically unfavorable (Futterman and Futterman, 1974; Rando and Chang, 1983).

Hence, we consider formation of 11-cis retinoids as a specific conversion related to the visual cycle and formation of 13-cis isomers a side reaction originating by different non-specific mechanisms. All 11-cis retinoid levels represent real 11-cis levels since they were measured as the corresponding retinol (cf chapter 2 section 2.1.4).

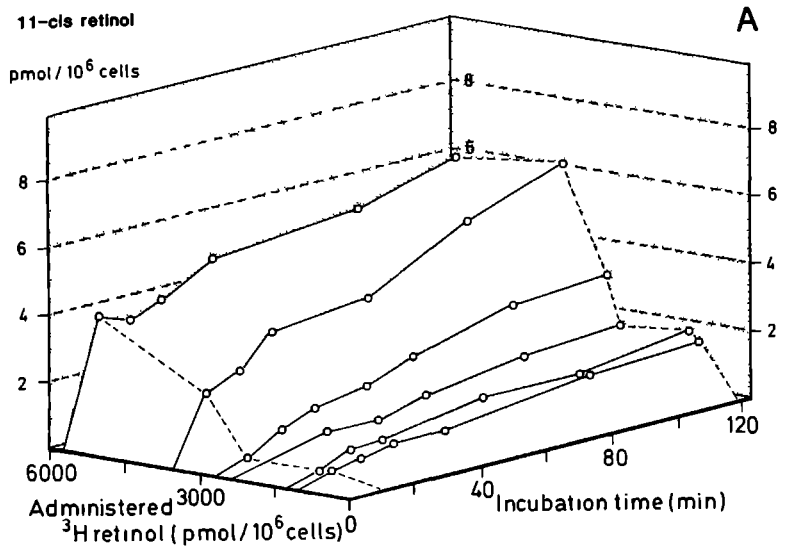
6.3.3.1. 11-cis retinol.

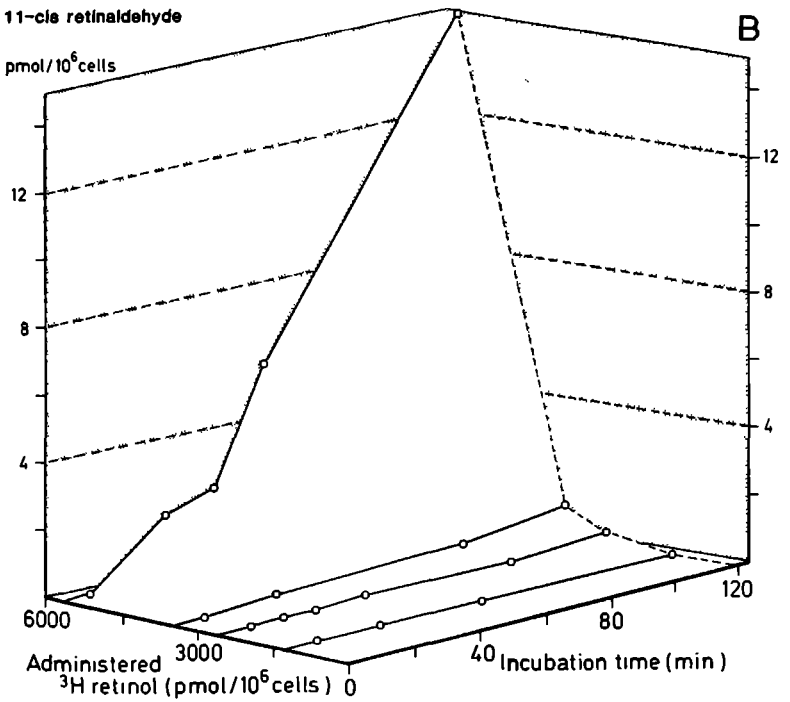
Labeled 11-cis retinol was only produced in low amounts and in a biphasic way (fig 6.5A). At lower labeled all-trans retinol levels, a slow formation of 11-cis retinol linear with time was observed. At higher all-trans levels, a relative rapid increase in 11-cis retinol level to a steady state of ca 6 pmol/10^6 RPE cells was displayed. This suggests that the isomerization is 'switched on' at a certain treshold intracellular retinol level. The steady state level of about 6 pmol labeled 11-cis retinol/10^6 RPE cells (compared to the unlabeled level of ca 10 pmol) in combination with the plateauing of the relative specific activity (fig 6.4) strongly suggests selective further processing of the labeled 11-cis retinol.

6.3.3.2. 11-cis retinaldehyde.

Although the concentration of labeled 11-cis retinaldehyde is underestimated due to incomplete extraction and aspecific isomerization during extraction, appreciable amounts were only detected at higher intracellular levels of labeled all-trans and 11-cis retinol.

Under these conditions the labeled 11-cis retinaldehyde level


11-cis retinol
A
pmol / 10^6 cells
8
6
4
2
6000
3000
0
Administered
3H retinol (pmol / 10^6 cells)
40
80
120
Incubation time (min)


11-cis retinaldehyde
B
pmol/10^6cells
12
8
4
6000
3000
0
Administered
3H retinol (pmol/10^6cells)
40
80
120
Incubation time (min)

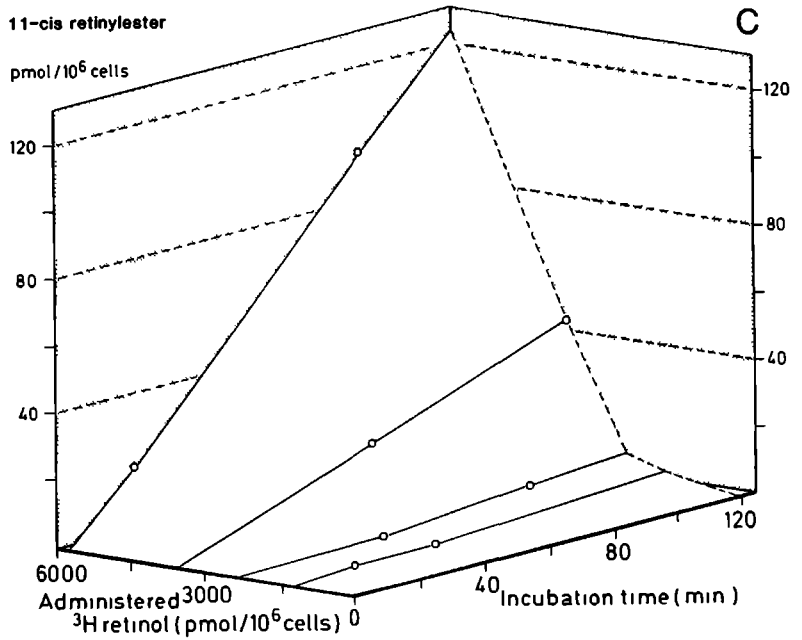


Fig 6.5
Cellular levels of labeled 11-cis retinoids in incubated retinal pigment epithelium cells in dependence of incubation time and labeled all-trans retinol supply.

In order to estimate real 11-cis retinoid levels, all cellular 11-cis retinoids were determined in the 'retinol mode' (cf chapter 2, section 2.2). The 11-cis retinoids were separated by means of HPLC and the label content in HPLC eluate fractions of 20 sec was determined by liquid scintilltion analysis. A: labeled 11-cis retinol; B: labeled 11-cis retinaldehyde; C: labeled 11-cis retinylester.

The RPE cells produced only low amounts of 11-cis retinoids. The formation of 11-cis retinaldehyde and 11-cis retinylester exhibits a remarkable dependence on the 11-cis retinol level.

increased rapidly and linearly with time (fig 6.5B): 11-cis retinaldehyde was formed at a rate of 0.13 pmol/min · 10^6 RPE cells and, after 120 min, exceeded the level of 11-cis retinol by about 200%.

A striking relation appears to exist between 11-cis retinaldehyde formation and 11-cis retinol level. Formation of 11-cis retinaldehyde only appears to pick up when the 11-cis retinol reaches a steady state level. The same phenomenon was apparent at the level of the relative specific activity (fig 6.4). These findings strongly suggests that isomerization preceeds oxidation and hence that isomerization takes place at the retinol stage. The apparent lack of interrelation between the formation of labeled all-trans retinaldehyde and labeled 11-cis retinaldehyde further supports this conclusion (fig 6.3C vs 6.5B).

6.3.3.3. 11-cis retinyl ester.

The formation of ^3H 11-cis retinylester followed a course very similar to that of ^3H 11-cis retinaldehyde. It could only be detected when the cellular 11-cis retinol level had reached a steady state (fig 6.5C). The rate of formation appeared to be strongly dependent on the administered retinol concentration. The maximal rate observed amounted to 1 pmol/min · 10^6 RPE cells and after 120 min of incubation a cellular level already 20-fold that of labeled 11-cis retinol. The relation with the steady state levels of 11-cis retinol is also apparent from the plot of the relative specific activities (fig 6.4).

The results suggest that esterification of 11-cis has a shorter time-lag, hence is switched on earlier than oxidation. However, this cannot be stated definitely, since the esterification rate is approximately 8-fold higher than the oxidation rate.

Little information is available on 11-cis esterification rates in vivo. In the frog, which contains approximately 2 x 10^6 RPE cells/eye, the formation rate of the 11-cis retinyl ester was estimated at 10 pmol/hr · 10^6 cells in the dark adapted animal (Fong et al., 1983). Illumination emptied the RPE store of 11-cis retinylester completely and increased the rate of 11-cis retinylester formation during subsequent dark adaptation by at least one order of magnitude to 170 pmol/hr · 10^6 cells (Bridges, 1976).

In the present experiments, the bovine eyes were exposed to daylight prior to enucleation, but were subsequently dark adapted. Due to the major differences in species and illumination history, a direct comparison is difficult to make, but the rate we observe in isolated bovine RPE (60 pmol/hr · 10^6 RPE cells) compares very well with the values reported for frog in vivo.

6.4. CONCLUSIONS.

6.4.1. GENERAL CONCLUSIONS.

The developed RPE incubation system appears to be an excellent in vitro system to study the vitamin A metabolism in the RPE cell:

(i) Uptake capacity.

The maximum rate observed (58 pmol retinol/min · 10^6 RPE cells) probably does not yet represent the rate maximal obtainable, but in 'in vivo' terms would already accomplish complete uptake of retinol, liberated upon a maximal retina bleach, in 17 min!

(ii) Esterification capacity.

Upon a maximal bleach, the esterifying machinery would be able to convert the incorporated retinol within 20 min. The maximal rate observed under the present conditions was 54 pmol/min · 10^6 RPE cells which is close to the uptake rate. This suggests that the retinol uptake was still the rate-limiting factor in the conversion of extracellular retinol into intracellular retinylester. The existence of a direct shuttle mechanism of transferring retinol to the esterification site might explain why freshly internalized retinol is esterified first.

(iii) 11-cis retinol oxidative capacity.

The overall V-max of the 11-cis retinol oxidoreductase in bovine RPE cells, as calculated from the data of Zimmerman et al. (1975), amounts to 1.1 nmol 11-cis retinaldehyde/min · mg total RPE protein. Since the oxidation and reduction rate are virtually equal (Lion et al., 1975), oxidation of 11-cis retinol could occur at a maximal rate of approximately 100 pmol/min · 10^6 RPE cells. This rate is approximately 3 orders of magnitude higher than the maximal 11-cis retinol oxidation rate determined in the experiments presented here (0.13 pmol/min · 10^6 RPE cells). This discrepancy cannot yet be solved, but it is conceivable that in particular the

important 11-cis retinaldehyde formation is under control of regulating factors, which have not yet been included in our incubation system. If in vivo the maximal attainable rate indeed would be about 100 pmol/min · 10^6 RPE cells, a full rhodopsin bleach in the bovine retina would require a period of dark adaptation of ca 2 hr for complete regeneration. A similar calculation for frog (ca 10 nmoles rhodopsin and ca 3 x 10^6 RPE cells per retina) yields dark adaptation times of 30 min. Hence this can easily account for the dark adaptation periods of 90 - 120 min have in vivo reported for frog (Bridges, 1976).

6.4.2. THE RPE CELLS SHOW ISOMERIZATION ACTIVITY!!

Our results present the first evidence that the RPE cell is indeed capable of isomerizing retinoids. Due to the rapid further processing of all-trans retinol into retinylester, the conversion rate of all-trans retinol into 11-cis could not be estimated. The formation of 11-cis retinaldehyde and 11-cis retinylester are limited by the levels of 11-cis retinol and likewise their rates can not be exactly calculated. Assuming, however, that at steady state levels of 11-cis retinol, the sum of the oxidation and the esterification rate approximates the rate of formation of 11-cis retinol, an approximate isomerization rate of 1.1 pmol/min · 10^6 RPE cells can be computed. Under these conditions, the regeneration of a fully bleached bovine retina would take almost 15 hr. However, these estimations most certainly represent suboptimal conditions. (i) The absence of an appropriate 11-cis retinaldehyde receptor (e.g. opsin) or another extracellular regulating factor might block 11-cis retinaldehyde transfer out of the cell and downregulate the isomerizing activity; (ii) the relatively poor extraction efficiency of the retinaldehyde might cause an underestimation of 11-cis retinaldehyde; and (iii) the rate of 11-cis retinylester formation might be a lower estimate due to the dark adaptation of the bovine eyes prior to the RPE cell isolation. Further experiments are required to shed light on these aspects.

Nevertheless, the clear dependence of 11-cis retinaldehyde levels on 11-cis retinol level and the absence of any relation between 11-cis retinaldehyde and all-trans retinaldehyde levels, unequivocally favor the concept that isomerization of all-trans

retinol preceeds oxidation. This conclusion is in agreement with a very recent report of Bernstein and Rando (1986) who, on basis of double label experiments, also suggest that isomerization takes place at the level of the all-trans retinol.

6.4.3. RETINOID PATHWAY IN THE RPE CELLS.

Fig 6.6 shows the retinoid pathway in the bovine RPE cell deduced from the present experiments. (i) All-trans retinol entering the RPE cells does not really mix with the endogenous

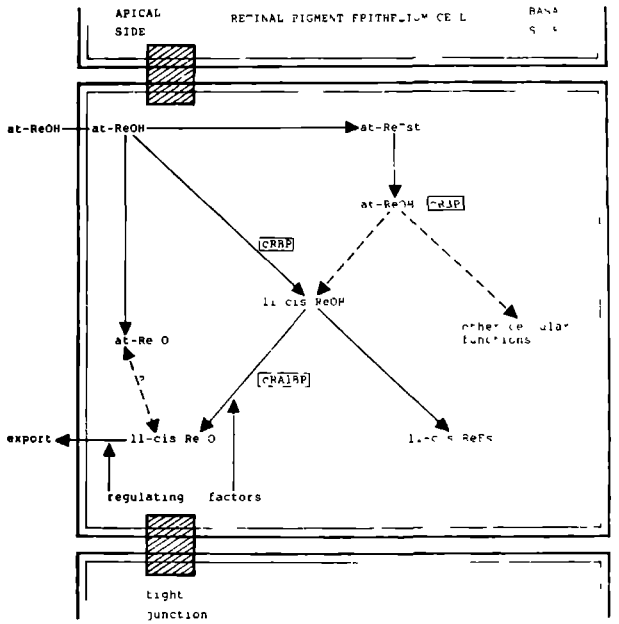


Fig 6.6.
Proposed metabolic pathways of retinol in retinal pigment epithelium.
After uptake, all-trans retinol is preferentially and rapidly converted into retinylester, which represents the storage form. cRBP is probably not involved in transfer of all-trans retinol to the site of esterification. The 11-cis retinol formation is triggered at a certain all-trans retinol level and/or flux. Subsequently 11-cis retinol is converted into 11-cis retinylester and 11-cis retinaldehyde probably in this order of preference. The formation of 11-cis retinaldehyde is probably regulated at the export and/or formation level. The lack of an appropriate 11-cis retinaldehyde receptor under these experimental conditions might explain the slow rate of 11-cis retinaldehyde formation.

all-trans retinol pool, but is immediately further processed mainly by esterification. Whether the cellular retinol binding protein is essential in this initial process as receptor and tranporter of all-trans retinol is doubtful (cf Maraini et al., 1985). (ii) The production of all-trans retinaldehyde is much slower than that of retinylester. Since no direct relation of the formation of all-trans retinaldehyde with other retinoids could be established, it might be considered as an overflow mechanism at high cellular retinol levels. (iii) The several lines of evidence discussed above support the concept that isomerization of retinol to take place before oxidation. (iv) Flexible handling of 11-cis retinoid store requires either compartimentalized storage of all-trans and 11-cis retinylester or a stereospecific retinylester hydrolase. Some evidence in favor of the latter has recently been presented (Blaner et al, 1985; Ishizaki et al, 1986).

6.4.4. IMPLICATIONS TO THE CURRENT MODEL OF THE VISUAL CYCLE.

A major implication of the presented findings on the current model of the visual cycle, is that the RPE's candidacy as the or a major retinoid isomerization site should be considered seriously. Although the estimated maximal isomerization rate was much to slow to carry dark adaptation, several suboptimal experimental conditions may affect the isomerization process particularly. The putative role of the Müller cells in the regeneration process will depend on the real maximal isomerization rate in the RPE. If this rate is indeed as low as measured, the RPE cannot carry regeneration and additional sites of isomerization are required. The RPE cells, low isomerization rates coupled to a large capacity, could provide back-up support in chromophore isomerization during peak requirements (high bleaching levels) and Müller cells could cover the need for 11-cis retinaldehyde at low illumination levels. This concept (postulated already in 1973 by Rotmans; cf thesis of Rotmans) of coexistence of two visual cycles: a long (via the RPE cells, retinol leaving the retina) and a short (via the Müller cells, retinol remains in retina) visual cycle requires further examination.

The present evidence strongly suggests that in RPE cells the isomerization step takes place at the level of the alcohol. This

conversion is likely to be catalyzed by an enzyme since in vitro retinol demonstrates low susceptibility to isomerizing agents in vitro (Groenendijk et al., 1980b; Bernstein et al., 1986a). Additional support for enzyme catalysis is provided by the fact that the isomerization into 11-cis is only observed in a higly organized system like intact RPE cells.

Chapter 7

PHAGOCYTOSIS OF RHODOPSIN BY BOVINE RETINAL PIGMENT EPITHELIUM CELLS IN VIVO AND IN VITRO AS EVALUATED BY A NEW QUANTITATIVE MOLECULAR ASSAY: THE DIFFERENTIAL IMMUNO ASSAY

7.1 INTRODUCTION.

Autoradiographical and morphological studies of the phagocytotic activity of RPE in vivo habe well documented the sequence of events taking place after the rod outer segment tis have been taken up in the RPE cells by endocytosis (Young and Bok, 1969; Ishikawa ano Yamada, 1970; Spitznas and Hogan, 1970; Herman and Steinberg, 1982b). Shortly following the endocytosis, the phagosome fuse with lysosomes (Herman and Steinberg, 1982b). Several major intermediate stages have been distinguished in the subsequent degradation process: (i) deformation of the disc stacking followed by (ii) disorganization of the membranes with loss of structural features in the phagosomal body and (iii) appearance of a granular matrix with a dense core (Ishikawa and Yamada, 1970; Spitznas and Hogan, 1970; cf Bok and Young, 1979). In the cause of this process the phagosomal bodies gradually decrease in size.

While the morphological changes accompanying phagocytic degradation of rod outer segment fragments in RPE have been well characterized, the molecular mechanism underlying this process is poorly understood. Little, if any, is known with respect to (phospho)lipids, which probably are processed very rapidly (Baker et al., 1986a). The protein fraction of the phagosomes is presumably degraded to the monomeric elements (amino acids and sugars). In vitro, the lysosomal fraction of bovine RPE is indeed capable of degrading rhodopsin, the major protein of the rod outer segment (Zimmerman et al., 1983; Hara et al., 1980, 1981, 1983). The major proteolytic activity of this fraction is represented by a cathepsin D-like enzyme (Zimmerman et al., 1983). The proteolytic fragmentation by cathepsin D has been studied by Regan et al. (1980). Their results, in combination with the well documented susceptibility of the C-terminus for proteolytic attack (cf Hargrave, 1982; de Grip and Margry, 1982) indicate that, the N-terminal part of opsin, which contains the two carbohydrate moieties is rather resistent to proteolysis. Most likely, a C-terminal part (2 - 4 kD) of opsin in cleaved off first.

In an attempt to describe the progress of phagocytosis in a more quantitative way, two morphological approaches are commonly used in

the literature: (i) characterization of the phagosome structure at the ultrstructural level or (ii) morphometry in order to determine the number of large phagosomes in the RPE cells. This number can be easily determined at the light microscopical level and is taken as an index for onset and progress of phagocytosis. In particular the latter approach has proven to be extremely useful in studies on the circadian rhythm of phagocytosis and its regulation by hormones, neurotransmitters and light (cf Besharse, 1982). However, both approaches obviously do not provide insight in the molecular events underlying phagosome processing and the accompanying opsin degradation.

A more direct approach, suitable for investigation of aspects of phagocytosis in vitro, has recently been described by Chaitin and Hall (1983a). Sequential immunolabeling, followed by dual-wavelength fluorometric analysis, is employed to distinguish between outer segments merely bound to and outer segments internalized by RPE cells. However, this procedure is very laborious and does does not provide information on the intracellular process of phagocytosis.

It occurred to us that the much higher susceptibility of the C-terminal region of (rhod)opsin for proteolysis, relative to the N-terminal region, in combination with the fact that antibodies directed towards these specific sites of opsin are available, might provide an immunochemical base to investigate the processing of phagosomal bodies. This could be done by measuring the progress of degradation of their major protein constitent (rhod)opsin. If the C-terminal part of opsin is indeed cleaved off at an early stage during proteolytic degradation, it is to be expected that the phagosomes should loose C-terminally located antigenic sites of opsin more rapidly than the N-terminally located epitopes. This principle may offer a more direct and more quantitative approach to follow phagocytosis of rhodopsin. First we will demonstrate that the above mentioned concept is indeed correct by means of immunohistochemical analysis of the intact RPE-retina complex both on light microscopic (immuno fluorescence) and electron microscopic level (single and double immunogold label technique). Subsequently, this concept will be materialized into a differential immuno assay, which allows us to analyse the process of phagocytosis on a molecular level both in vivo, following cell isolation, and in

vitro during short-term incubation of isolated cells. This procedure entails immunochemical determination of opsin 'in statu digestandi' by a differential procedure: a monoclonal antibody, directed towards the C-terminal part of opsin, is used to measure intact, non-degraded opsin and a polyclonal antiserum, mainly directed towards the N-terminal but without activity towards the C-terminal part is used to measure total (intact + fragmented) opsin. Finally, the suitability of incubated bovine RPE cells, as a mammalian in-vitro system to study phagocytosis, will be discussed.

7.2. MATERIALS AND METHODS.

7.2.1 MATERIALS.

The monoclonal antibody 1D4 was a generos gift of dr R. Molday (University of British Columbia, Vancouver, Canada). This antibody recognizes the final residues 340-348 in the C-terminal region of (rhod)opsin (MacKenzie et al., 1984) and will further designated 'Anti-C'. The 'Anti-N' antiserum was raised in rabbits following standard procedures in our laboratory (Margry et al., 1983) using proteolytically (proteinase K) treated rhodopsin (Rh-PK) as immunogen. Rh-PK lacks the most susceptible proteolytic sites (C-terminal and part of 5 - 6 loop; cf Hargrave, 1982; de Grip, 1985). The resulting antiserum indeed shows strong activity towards an opsin N-terminal peptide (2-32) immobilized onto BSA and no activity towards a similarly immobilized C-terminal peptide (332-348) or a 5-6 loop peptide (231-252).

Rabbit anti-mouse immunoglobulin and swine anti-rabbit immunoglobulin conjugated to FITC were obtained from Dakopatts (Glostrup, Denmark), goat anti-rabbit immunoglobulin conjugated to 5 nm gold particles of 10 nm gold particles were obtained from Janssen Pharmaceutica (Beerse, Belgium) and swine anti-rabbit immunoglobulin conjugated to horse-radish peroxidase and rabbit anti-mouse immunoglobulin conjugated to horse-radish peroxidase from Dakopatts. Lowicryl K4M was obtained from Polyscience Inc. (Warrington, Pennsylvania, USA), glutaraldehyde and formaldehyde from E.M. Sciences (Washington, USA). Polystyrene, 96-wells flat-bottom microtiter plates were obtained from Flow Laboratories (Irvine, Scotland) and o-phenylenediamine, Ammonyx LO and bovine serum albumine from Sigma Chemical Co. (St Louis, Missouri, USA).

7.2.2. METHODS.

7.2.2.1. Immunohistochemistry at the light microscopic level.

Immediately following enucleation of bovine eyes the anterior part and vitreous were removed. The eyecup was soaked for ca 4 hr in Bouin's fixative (a mixture of saturated picric acid, formaline (37%) and glacial acetic acid; 15:5:1, v/v/v). Pieces of the tapetal area, containing retina, RPE and choroid, were cut out and extensively dehydrated in 100% acetone (4 steps of 1, 2, 2, and 4 hr). Subsequently, the pieces of tissue were softened in methylbenzoate (1.5 hr; room temperature), immersed in xylene (2 x 0.5 hr; room temperature) and embedded in Paraplast (12 hr at 60^{o}C).

Sections of 3 µm were deparafinized in xylene, washed with ethanol and rehydrated in PBS (10 mM sodium phosphate; 150 mM NaCl; pH 7.2). The sections were then immunolabeled by incubation for 1 hr at room temperature with 100 µl of either a solution of the Anti-C antibodies , or Anti-N antiserum of preimmune serum (all diluted 200-fold in PBS-1%BSA). Thereupon the sections were thoroughly washed in PBS for 10 min. In case of the monoclonal Anti-C antibodies, a 'bridge-reaction' with rabbit anti-mouse antiserum (1/100 in PBS-1%BSA) was then performed first (1 hr; room temperature). Subsequently, all antibody complexes were stained by incubation for 1 hr at room temperature with swine anti-rabbit conjugated to FITC (1/200 in PBS-1%BSA). The immunostained sections were immediately analyzed on a Leitz fluorescence microscope, equipped with an excitation/emission set up for FITC.

7.2.2.2. Immunohistochemistry at the electron microscopic level.

Immediately following enucleation of bovine eyes the anterior part and vitreous was removed. The eyecups was fixated for 4 hr at room temperature by immersion in a solution of 3% paraformaldehyde, 1% glutaraldehyde, 0.2% picric acid in 0.1 M sodium cacodylate (pH 7.35). Tapetal pieces, containing the retina, RPE and choroid, were cut out, rinsed in 0.1 M sodium cacodylate (pH 7.35) and dehydrated trough a graded series of ethanol. The pieces of tissue were then impregnated with a graded series of Lowicryl K4M: initially in ethanol:Lowicryl K4M cocktails (resp. 2:1, 1:1, 1:2, each for 1 hr at room temperature) and then 100% Lowicryl K4M overnight.

Polymerization of Lowicryl K4M was achieved by illumination with UV light (Philips Black-Light 360 at a 10-15 cm distance) for one day at 4°C, followed by 4 days at room temperature. Ultrathin sections (silver: 60 - 80 nm) were cut with a diamond knife (Diatom, Schwitzerland), collected on copper grids, covered with supporting film (Formvar) and subjected to the antiserum incubations.

Immunolabeling of the sections on grids with the anti-opsin antisera and immunogold conjugated second antibodies was optimized with respect to dilution and incubation time. This resulted in the following procedures.

The sections were incubated for 1 hr at room temperature with the anti-opsin antibodies or preimmune serum (50 µl; diluted 200-fold in PBS-1%BSA). After extensive rinsing with PBS-1%BSA the immunocomplexes were stained with one of the following second antibodies. (i) goat anti-rabbit conjugated to 5 nm gold particles (1/50 in PBS-1%BSA), (ii) rabbit anti-mouse (1/100 in PBS-1%BSA) ('bridge reaction'), followed by goat anti-rabbit conjugated to 5 nm gold particles, or (iii) goat anti-mouse conjugated to 10 nm gold particles (1/5 in PBS-1%BSA). These incubations visualized the Anti-N immunocomplexes via 5 nm gold particles (i) or the Anti-C immunocomplexes via 5 nm gold particles (ii) or 10 nm (iii) gold particles. The immunogold labeled sections were rinsed with PBS-1% BSA, post-fixated with 1% glutaraldehyde in 0.05 M sodium cacodylate (15 min at room temperature) and stained with OsO_4 vapor (2 min), uranylacetate (15 min; room temperature) and lead citrate (5 min, room temperature). The sections were finally examined in a Philips EM 300 or EM 301 electron microscope.

Single label experiments with 5 nm gold particles resulted in high label density in rod outer segments both for Anti-C (1470 $\pm$ 210 gold particles/μm^2) and for Anti-N (1300 $\pm$ 110 gold particles/μm^2; n=3; + SE). All parts of the retina, except for phagosomes in the RPE, only showed background labeling (40 $\pm$ 10 particles/μm^2). Preimmune sera yielded densities of less than 50 gold particles/μm^2 troughout the retina.

In double-label experiments, the order of incubation with the second antibodies appeared to the affect label density strongly. Simultaneous incubation with both anti-opsin antisera followed by a first labeling step with goat anti-rabbit conjugated to 5 nm gold

particles, almost completely abolished the second labeling with 10 nm immunogold particles. Reproduceble results were only obtained by a simultaneous incubation with both Anti-C and Anti-N antibodies followed by simultaneous labeling with both goat anti-rabbit conjugated to 5 nm gold and goat anti-mouse conjugated to 10 nm gold. In this way the following label densities in ROS were obtained (n=3; + SE): Anti-C: 280 + 10 gold (10 nm) particles/μm^2; Anti-N: 720 $\pm$ 40 gold (5 nm) particles/μm^2. The label density with Anti-N was approximately half that obtained with single labeling and the Anti-C label density was about 5-fold reduced compared to single-label experiments. The latter reduction was probably partially due to the considerably lower particle density in the 10 nm immunogold batch relative to the 5 nm batch. However, taking into account that these double label experiments are performed against a single protein of relatively low M_r (40 kD), the results are quite satisfactory and no further attempts were made to optimize the labeling procedure.

7.2.2.3. Cell isolation.

Isolation of bovine RPE cells was performed via the perfusion technique as described in chapter 3 (section 3.2.2.1 and 3.2.2.2).

7.2.2.4. Determination of cell density.

Cell density was determined by means of a DNA assay carried out as described in chapter 2 (section 2.2.2).

7.2.2.5. Cell incubation.

Short-term incubations of the isolated RPE cells were carried out as described in chapter 5 (section 5.2.2.3).

7.2.2.6. Electro-immunoblotting.

SDS-PAGE, electro-immunoblotting and visualization of antibody-antigen complexes was performe as described in chapter 2 (section 2.3). Antisera were diluted 200-fold in TBS-1%gelatin.

7.2.2.7. Development of a differential immunoassay (DIA) for the determination of opsin 'in statu digestandi'.

The amount of opsin in RPE cell preparations was assayed by means of an ELISA antigen inhibition assays using either the

monoclonal Anti-C antibody (1D4) specific for the C-terminal part of opsin (MacKenzie et al., 1984), or the Anti-N antiserum specifically reactive with carbohydrate containing N-terminal fragments of opsin (de Grip, 1985).

I. The single antigen inhibition assays.

Optimization of the inhibition ELISA with Anti-C or Anti-N antibodies was carried out along the general lines, described by Schalken and de Grip (1986). Only the final experimental conditions will be given.

A. Opsin coating. The optimal opsin coating was achieved, both for Anti-C and Anti-N antisera, by incubation of the microtiter plate with a solution of 16 pmoles opsin/ml in PBS-0.0006% Ammonyx LO (150 µl/well) for 16 hr at 4^{o}C. The opsin solution was prepared by dissolving 25 µl of a suspension of purified rod outer segment membranes (50 µM rhodopsin) in 25 µl PBS-2%Ammonyx LO, followed by illumination (10 min; 300 W) and 1600-fold dilution of the bleached solution with PBS.

B. Assay with Anti-C antibodies. The Anti-C antibody solution (4 mg/ml) was diluted 4000-fold with PBS-0.1%BSA-0.01%Ammonyx LO. The inhibiting antigen was suspended in PBS-0.1%BSA-0.01%Ammonyx LO and sonicated (3 times, 10 sec, Branson sonifier equipped with microtip, output 10 W).

C. Assay with Anti-N antiserum. The Anti-N antiserum was diluted 3000-fold in PBS-0.1%BSA-0.1%Ammonyx LO. The inhibiting antigen was suspended in PBS-0.1%BSA-0.01%Ammonyx LO and sonicated (3 times, 10 sec). Subsequently, Ammonyx LO (10% in PBS-0.1%BSA) was added to a final Ammonyx LO concentration of 0.1%.

D. Calibration. A suspension of purified rod outer segments in PBS-0.1%BSA-0.01%Ammonyx LO (25 µM rhodopsin) was illuminated (10 min, 300 W) and diluted to 500 nM rhodopsin by addition of 50 volumina of PBS-0.1%BSA-0.01%Ammonyx LO. After sonication (see above) one part was used as such in the inhibition assay with Anti-C antibodies and the other part was adjusted to the conditions required for the inhibition assay with Anti-N antiserum by addition of Ammonyx LO (see section C). The assay procedures followed those described by Schalken and de Grip (1986). The resulting calibration curves are displayed in fig 7.1.

II. The differential immunoassay (DIA).

For determination of opsin 'in statu digestandi' in a population

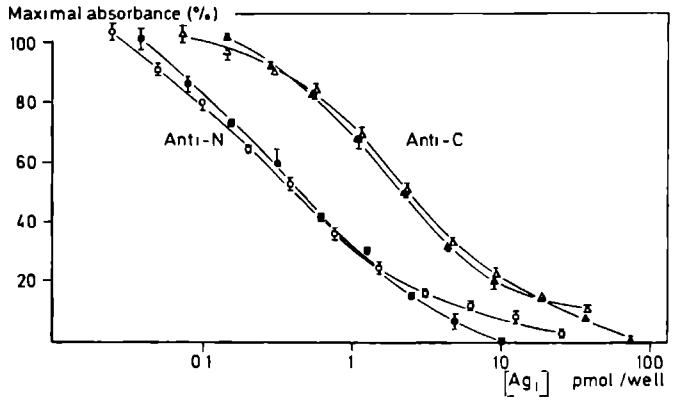


Fig. 7.1
Comparisom of the avidity of Anti-N antibodies or Anti-C antibodies for intact opsin and for opsinoids in bovine RPE.

Inhibition ELISA curves using Anti-N antiserum (triangles) or Anti-C antibodies (circles) with bovine ROS membranes (open symbols) or bovine RPE-homogenate (filled symbols) as inhibiting antigen. Coating was performed with ROS-membranes. For experimental details see section 7.2.2.7. The percentage of the maximal absorbance was plotted semi-logarithmically against the amount of opsin added. Since the amount of opsinoid in the RPE cannot be determined in another way, absolute positioning of these curves along the X-axis cannot be done. Therefore, the inhibition curves for these samples are placed with optimal overlap over the corresponding ROS membrane curves. It is evident, that the slope of the corresponding ROS and RPE curves are not significantly different. This is the essential element since it demonstrated that the avidity of the antisera for ROS-opsin and RPE-opsinoids is similar and hence that ROS-opsin can be used for calibration on basis of which RPE-opsinoid can be quantitated.

of intact and fragmented opsin, it is obligatory to be able to discriminate between intact and digested opsin. Intact opsin was determined with the Anti-C antibody which only reacts with non-fragmented opsin (fig 7.8). Total opsin (intact + fragmented) was determined by means of the Anti-N antiserum which does not react with epitopes on the C-terminal part of opsin. The latter aspect was confirmed by comparing the inhibition curves obtained with Anti-N antiserum and intact opsin or proteolytically (thermolysine or proteinase K) fragmented opsin (fig 7.2). Since all inhibition antigens achieve complete inhibition and the resulting inhibition curves completely coincide, the conclusion is allowed that the Anti-N antiserum indeed does not recognize sites

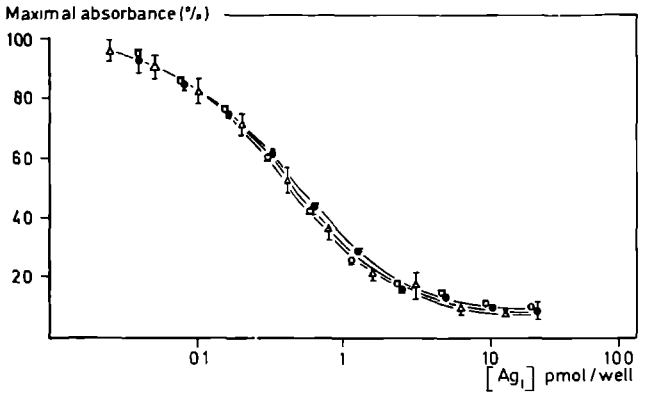


Fig. 7.2
Comparison of the avidity of Anti-N antibodies for intact opsin and for proteolytically digested opsin.

Inhibition ELISA curves using Anti-N antiserum with intact ROS membranes (triangles), proteinase K digested ROS membranes (open circles) or thermolysin digested ROS membranes (filled circles) as inhibiting antigen. Coating was performed with ROS membranes. For experimental details see section 7.2.2.7. The percentage of maximal absorbance was plotted semi-logarithmically against the amount of opsin added. For all inhibition antigens the absolute amount has been determined spectroscopically.

in opsin, which are easily removed by proteolytic cleavage (C-terminal; 5-6 loop).

A. Preparation of inhibiting antigen from RPE cell samples. Isolated or incubated RPE cells were harvested by centrifugation (Eppendorf 5414 centrifuge; 5 sec; 8,000 x g) and stored as a pellet at -20°C. Just before the assay, RPE cells were suspended in PBS-0.1%BSA-0.01%Ammonyx LO to a concentration of 2 $x10^6$ cells/ml by mixing on a Vortex mixer. The resulting cell suspension was divided in two equal parts. One part was used as it was for the inhibition assay with Anti-C antibody, the other part was used for the inhibition assay with Anti-N antiserum and its Ammonyx LO concentration was therefore adjusted to 0.1% as described above. After a sonication step (3 x 10 sec), both mixtures were used directly in the assay. An example of both antigen inhibition is shown in fig 7.1.

B. Calculation of opsin 'in statu digestandi'. The inhibition assay with the Anti-C antibody allows to calculate the amount of intact opsin present in the RPE samples using the specific

calibration curve in fig 7.1. In a similar way the inhibition assay with the Anti-N antiserum allows to calculate 'total opsin' (=intact + fragmented opsin). Subtraction of intact opsin from total opsin yields the amount of opsin 'in statu digestandi' present in the RPE cell preparation.

7.3. RESULTS AND DISCUSSION.

7.3.1. PHAGOCYTOSIS IN VIVO: DOUBLE LABEL IMMUNOHISTOCHEMISTRY DEMONSTRATES THAT DIGESTION OF (RHOD)OPSIN STARTS AT THE C-TERMINAL AS AN EARLY EVENT DURING PHAGOSOME PROCESSING.

As mentioned before, phagosome processing in the RPE cells of amphibian and mammalian species has been characterized by transient stages in ultrasructure of the phagosome as well as by the circadian pattern in phagosome number. The beginning of the light period is rapidly followed by an initial steep increase in the number of phagosomes, which subsequently decreases in several hours to a steady background level (LaVail, 1976; cf Besharse, 1982). By now, site specific antibodies against rhodopsin have become available. This prompted us to investigate whether immunohistochemical analysis could provide more specific information on these morphological stages in phagosome processing, e.g. relate them to molecular processes like the digestion of opsin. Hereto, we employed immunohistochemistry at the light microscopical level for morphometric quantitation of labeled phagosomes as well as at the electron microscopical level in an attempt to correlate ultrastructure with labeling pattern. For immunolabels we used the monoclonal Anti-C antibody or the Anti-N antiserum. Visualization of the immunocomplexes was accomplished by fluorescent (light microscopy) or gold conjugated (electron microscopy) second antibodies.

7.3.1.1. The number of phagosomes suggests photoperiodism in cattle.

In mammalian and avian species phagocytosis of the rod outer segment tips seems to follow the same circadian rhythm: a phagocytotic outburst shortly after the onset of the light yields a massive increase in the number of phagosomes and phagosome

degradation is the predominant subsequent process. This circadian rhythm is well characterized in rat and opossum. Although it is expected to be similar in cattle, no data are available in the literature. A major problem is that the light-dark cycle of cattle is not easyly accessible to experimental manipulation.

As a comprimise we therefore took the time of sunrise as beginning of the light period. Animals were slaughtered at various times through the day and their eyes were processed immediately for immunhistochemical analysis. The number of phagosomes which produced a positive reaction with Anti-C or Anti-N antibodies were counted in a defined arbitrarial area of RPE cells. A plot of this number of phagosomes versus 'light time' is shown in fig 7.3. The Anti-N part of this plot resembles the descending phase of a circadian profile (LaVail, 1976; Herman and Steinberg, 1982a). No clear indication for a rising phase in the phagocytotic outburst was obtained. This is probably due to the poorly defined light-dark cycle in combination with the fact that animals slaughtered very early in the light period were not available. Very remarkably, the number of Anti-C positive phagosomes declined much more rapidly

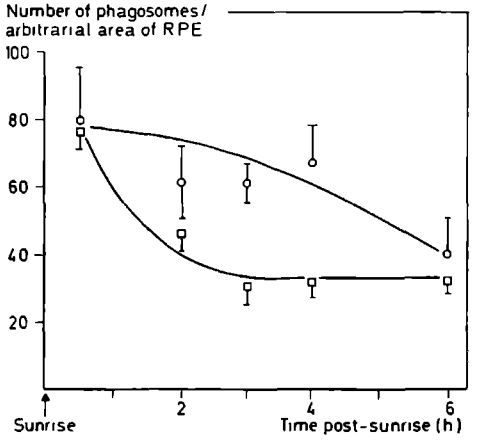


Fig. 7.3
Number of phagosomes in bovine RPE in dependence of the time interval in the light period (time post-sunrise).
Anti-C reactive phagosomes (squares) or Anti-N reactive phagosomes (circles) were visualized by immunofluoromicroscopy on parallel sections. The number of phagosomes was counted in a constant arbitrarial area within RPE and is plotted against the time post-sunrise. The data points represent the average of 2 to 4 determinations on 2 samples. Bars indicate standard errors.

than that of the Anti-N positive phagosomes, although the same final level was reached. In fact, the phagosomes almost completely loose their Anti-C reactivity before a marked loss in Anti-N reactivity is obvious. These findings indicate that rapid C-terminal cleavage of opsin occurs in the early stage of phagosome processing and overall precedes digestion of the N-terminal parts of opsin.

7.3.1.2. Ultrastructural morphology of phagosomes in relation to opsin degradation.

In rat and oppossum the ultrastuctural changes accompanying phagosome processing have been well documented (Herman and Steinberg, 1982a,b). These changes can already be recognized shortly following internalization of the nascent phagosomes. Hence, it seemed appropriate to investigate whether these changes could be related to the stages in opsin digestion described above using ultra-immunohistochemistry. A major advantage of this approach is that a double-label technique can be applied using gold particles of different size, so that the reactivity of the phagosome towards the Anti-C (10 nm gold) or the the Anti-N (5 nm gold) sera could be analyzed simultaneously on the very same phagosome.

Bovine eyes were obtained from animals slaughtered at various times through the day and immediately processed of ultra immunohistochemical analysis (fig 7.4 and table 7.1).

Although, the immunohistochemical approach does not allow the fixation and staining procedures necessary for detailed morphological analysis on the structural level, the developed procedure provides sufficient detail so as to distinguish three major stages of phagosome degradation: phagosome type I (fig 7.4B), which closely resembles rod outer segment ultrastructure (fig 7.4A); phagosome type II (fig 7.4C), which has lost the characteristic disc membranes structure and has a more homogenous appearance; and phagosome type III (fig 7.4D), which demonstrates almost no ultrastructure except for a dense granular core. The morphology of these stages agree on this level of detail very well with the major transient stages described before (Ishikawa and Yamada, 1970; Spitznas and Hogan, 1970; cf Bok and Young, 1979; Herman and Steinberg, 1982a,b). The conversion of phagosome type I to type II appeared to be due to fusion of the nascent phagosome

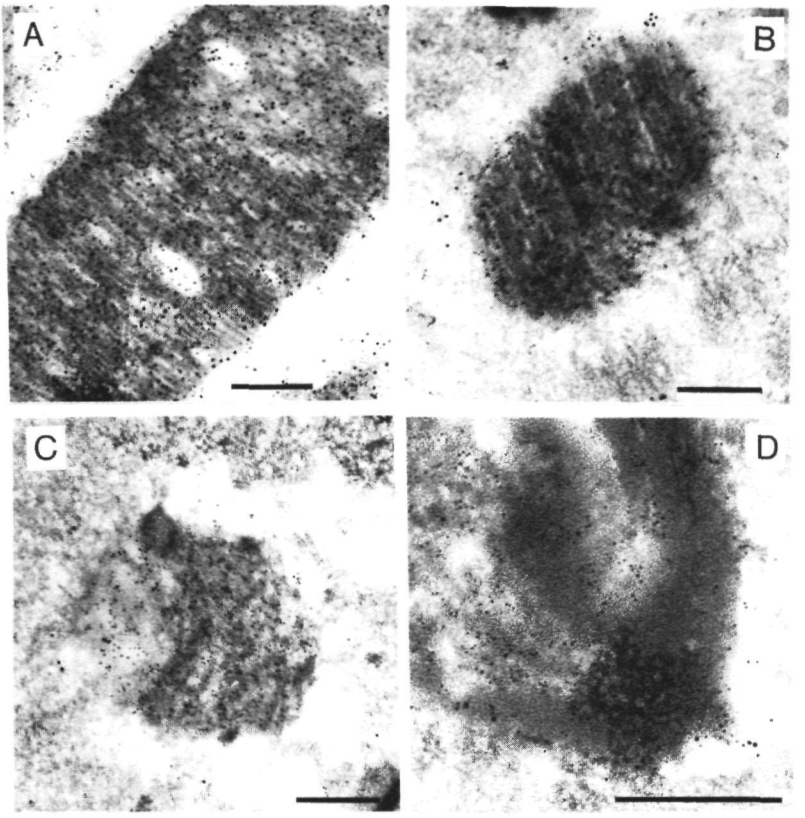


Fig. 7.4
Immunogold double-labeling of ROS and the three types of phagosomes.
Fixated eyecup preparations were embedded in Lowicryl K4M and subjected to double-immunolabeling (section 7.2.2.2). The 10 nm gold particles represent Anti-C reactivity. The 5 nm particles represent Anti-N reactivity. Typical pictures are shown of: A. ROS; B. phagosome type I; C. phagosome type II; phagosome type III. Bar represents 0.2 µm.

with lysosomes, a process which is effectuated within 30 min after endocytosis (Herman and Steinberg, 1982b).

The label density of the three types of phagosomes was quantatively determined for a total of 12 phagosomes, at three time points in the light period (table 7.1, fig 7.5). The 10 nm particles, representing Anti-C reactivity and the 5 nm particles, representing the Anti-N reactivity, could be easily distinguished. In order to evaluate, whether the labeling procedure gave reproducible results and whether the various experiments could be

TABLE 7.1 PHAGOSOME PROCESSING IN BOVINE RPE (DOUBLE IMMUNOLABELING WITH ANTI-C AND ANTI-N)[a]

ORGANEL[b]	TIME OF ENUCLEATION AFTER SUNRISE					
	0.5 hr		3.5 hr		6.5 hr	
	ANTI-C	ANTI-N	ANTI-C	ANTI-N	ANTI-C	ANTI-N
ROS (n=12)	277±60	662±62	281±44	743±103	292±33	748±65
PHAGO I (n)	273±46 (9)	690±169	213±83 (2)	889±304	316±40 (6)	754±79
PHAGO II (n)	35±18 (3)	789±32	63±18 (10)	1087±92	40±26 (3)	1022±98
PHAGO III (n)	N.D.		N.D.		4±6 (3)	205±170

a: Label density in particles/μm^2 (mean ± SD), of 10 nm gold (Anti-C antibodies) and 5 nm gold (Anti-N antibodies) of ROS and phagosomes. The latter could be subdivided ultrastructurally into three degradation stages: phago I, II and III. The number of label determinations per experiment are presented between parentheses.

b: Note that these determinations do not reflect phagosome densities! The sample collected 6.5 hr after sunrise had a much lower phagosome density and a large area had to be screened to find 12 phagosomes (cf fig 7.3). Background label densities, determined on tissue and non-tissue parts of the sections, amounted to (n=7): anti-C: 16 ± 7 and anti-N: 28 ± 10.

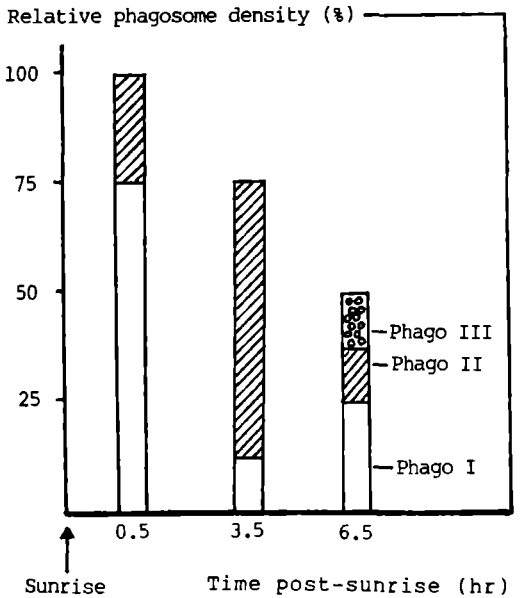


Fig. 7.5
Distribution of type of phagosomes and relative phagosome density at various time intervals of light period (post-sunrise) during phagocytosis in vivo

The relative Anti-N reactive phagosome dersity is deduced from data in fig 7.3 and is combined with the distribution of phagosome type (table 7.1).

mutually compared, the label density of the rod outer segments, which should remain constant in time, was determined as well. The label density of the rod outer segment (fig 7.4A, table 7.1) showed no significant differences throughout all experiments. Furthermore, no difference in density for either Anti-C or Anti-N reactivity could be detected between basal and distal part of the outer segment. Hence, the developed labeling procedure worked very reproducibly. The label density in the phagosomes showed a very interesting pattern (table 7.1). First of all, it should be noted that the absolute phagosome density decreased at least twofold between 0.5 and 6.5 hr post-sunrise, in agreement with the light microscopic observations (fig 7.3). More importantly, very significant differences in label density for the three types of phagosomes were detected (table 7.1). Type I yields a label density, both for Anti-C and Anti-N, which is very comparable to intact rod outer segments. Conversion of type I into type II is accompanied by a precipitous drop in Anti-C label density and a definit increase in the Anti-N label density. Conversion to type III concurs with removal of the last tracer of Anti-C reactivity, while now the Anti-N label density was also markedly reduced. These observations completely corraborate and extend the light microscopic analysis. Type I phagosome represent freshly internalized rod outer segment material. Rapid subsequent fusion with lysosomes produces type II phagosomes and in the course of this process the digestion of opsin is initiated, which first of all quickly removes Anti-C reactive sites. The increase in Anti-N label density might reflect a 'concentration effect', due to shrinkage of the phagosome in the course of the digestion and removal of outer segment material, or be due to relief of competition between antibodies due to absence of Anti-C reactive sites, so that Anti-N label density would approach that of single label experiments. Single-label experiments showed no increase on Anti-N label density upon conversion of type I phagosome into II (not shown) and hence support the second explanation. The N-terminal fragments are digested much slower and are still detectable in type III phagosomes, 6.5 hr post-sunrise. The presence of type I phagosomes at that stages however indicates that a low level of continuous phagocytosis is maintained.

These results clearly demonstrate that C-terminal digestion of opsin is complete before significant digestion of the N-terminal part is obvious. Furthermore, this C-terminal cleavage occurs at an early stage following the endocytosis of the rod outer segment tips and can be considered to be a very early event in phagocytosis. Consequently, the opsin species present during phagosome processing is mainly represented by a collection of digestion products (opsinoids) which all miss at least their C-terminal part and which we will call opsin' in statu digestandi'.

7.3.2. PHAGOCYTOSIS IN VIVO: OPSIN DIGESTION ANALYZED BY A NEW ASSAY, THE DIFFERENTIAL IMMUNO ASSAY (DIA).

The use of the number of phagosomes as a parameter in studies on phagocytosis presents several experimental problems. For instance it is difficult to discriminate between outer segment fragments inside and outside the RPE cells. Further, the size and the type distribution of the phagosomes may vary considerably and thus the number is not a suitable parameter to quantify phagocytized opsin or analyze the kinetics of phagocytotic opsin digestion.

The immunohistochemical studies, presented above, allow the conclusion that digestion of the C-terminal opsin is a very early, if not the first, event in the phagocytotic proces and intact, Anti-C reactive, opsin is only present outside the RPE cell or in very shortly internalized phagosomes. On this basis we developed an alternative approach to quantify opsin 'in statu digestandi'. Intact C-terminal containing opsin can be selectively assayed by means of an immuno assay using the monoclonal Anti-C antibodies. The N-terminal containing opsinoids, which represent total opsin, i.e. the sum of intact opsin and opsin 'in statu digestandi' can be determined via an immuno assay using Anti-N antiserum. Hence, subtraction of the amounts of anti-C positive opsin from the amount of anti-N positive opsinoid directly yields the amount of opsin 'in statu digestandi' which goes through phagocytotic processing in the RPE cell.

This concept has been developed into a differential immuno assay, based on two complementary inhibition ELISA's with either Anti-C or Anti-N antibodies (cf materials and methods). It has been used to measure opsin 'in statu digestandi' in bovine RPE cells

isolated from animals slaughtered at various time-points in the light period.

7.3.2.1. Determination of opsin 'in statu digestandi' in freshly isolated bovine retinal pigment epithelium cells.

Bovine RPE cells were isolated within approximately 1.5 hrs after enucleation of the eyes from cattle slaughtered at various times through the day and processed for the DIA measurements as described under Materials and Methods. Fig 7.6 shows a plot of the amount of Anti-C and Anti-N reactive material versus time. The amount of intact opsin varies between 200 and 300 pmoles/10^6 RPE cells and only shows a slight decrease in time. This opsin population probably represents a steady background due to

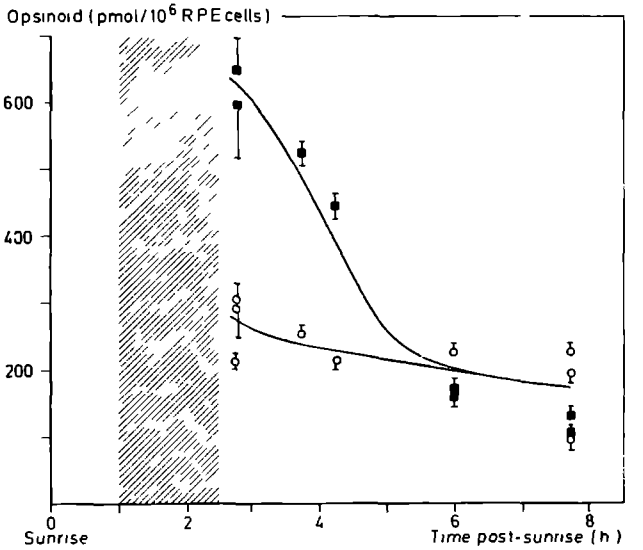


Fig. 7.6
The course of opsin degradation during phagocytosis in bovine RPE in vivo.

The opsin populations in bovine RPE preparation isolated at various time points in the light period (post-sunrise) were determined with DIA and expressed as pmol per 10^6 RPE cells. Open circles represent intact opsin. Filled squares represent total opsinoids. The area between these curves represent opsin 'in statu digestandi'. Data points represent 2 measurements on 1 sample. Error bars represent standard deviation. The shaded area represents the time between enucleation of the eyes and preparation of the isolated RPE cells for DIA analysis.

contamination of the RPE preparation with ROS (ca 2% of total retinal opsin) in combination with a dynamic equilibrium of internalization and degradation of opsin. The latter process probably reflects the low level of continuous background phagocytosis, which probably is also responsible for the occurrence of phagosome type I in the RPE cells of eyes collected 6.5 hr post-sunrise (table 7.1). The Anti-N reactive population sharply declines in time, with a time course comparable to that of the number of phagosomes (fig 7.3) and the immunolabel pattern (table 7.1). The final level is the same as for the Anti-C reactive population and hence represents intact opsin.

The amount of opsin 'in statu digestandi' can be calculated at each time point from the difference between the total opsinoid curve (Anti-N positive population) and the intact opsin curve (anti-C positive population) and maximally amounts to ca 500 pmol/10^6 RPE cells. The daily load of opsin to be phagocytized in the bovine retina expected to approach approximately 10% of the total ocular rhodopsin content (ca 50 nmoles) and would amount to about 1000 pmol/10^6 RPE cells. In analogy with the process of phagocytosis in other species, it may be expected that ca 80% of the daily load is internalized during the phagocytosis outburst shortly after the beginning of the light period (cf Besharse, 1982). Taken into account that, since early time points were lacking, the amount of phagocytized opsin is probably underestimated, we conclude that the results of the DIA are in fair agreement with the other phagocytotic assays and with theoretical calculations.

Due to the poor definition of the light cycle of the bovine animal and the time required to isolate the RPE cells (indicated by the black box), it was again not possible to collect data points during the rising phase of phagocytosis. Hence, the rate of endocytosis could not be calculated. The slope of the declining phase fig 7.6 yields an apparent degradation rate of approximately 200 pmol opsin/10^6 RPE cells $\cdot$ hr. This rate is sufficient to degrade the complete daily load of opsin for phagocytosis within 5 hr, which compares well with a degradation time of ca 4 hr determined in rat and opossum (LaVail, 1976; Herman and Steinberg, 1982a).

7.3.2.2. Opsin fragmentation pattern during phagocytotic processing in vivo.

The fragmentation pattern of opsin during phagocytosis in vivo was analyzed by subjecting bovine RPE cells, isolated throughout the light period, to SDS poly-acrylamide-gel- electrophoresis followed by electro-immunoblotting. Anti-C antibodies were used to visualize intact opsin only, Anti-N antiserum to visualize intact opsin as well as fragmentation products. The fragmentation pattern, detected by the anti-N antibodies is shown in fig 7.7. Four major bands are apparent: a band of 40 kD, representing intact opsin, and three degradation products of 33, 25, and 18 kD. The level of these proteolytic fragments decreases in time and only the smallest one clearly persists into the more advanced stages of phagocytosis. The intact opsin band probably again represents ROS contamination. Anti-C antibodies only reacted with the 40 kD band (not shown). The observed fragmentation pattern is comparable to that described by Regan et al. (1980). The minor differences observed are probably due to differences in electrophoresis conditions.

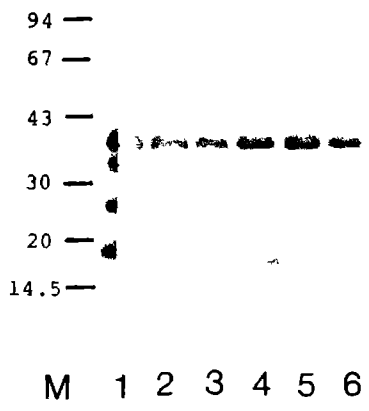


Fig. 7.7
Fragmentation pattern of opsin during phagocytosis in bovine RPE in vivo.
Samples of bovine RPE cells (6×10^4 cells/lane) isolated at various times in the light period (post-sunrise), were subjected to electro-immunoblotting. Opsinoids are detected by means of Anti-N antiserum (diluted 500-fold). RPE cells were isolated 1.15 hr (lane 1); 2.45 hr (lane 2); 3.45 hr (lane 3); 4.45 hr (lane 4); 6.45 hr (lane 5); 7.45 hr (lane 6) post-sunrise. Preimmune sera gave no detectable reaction. M represents the molecular weight markers (MW x 10^{-3} kD).

7.3.3. PHAGOCYTOSIS IN VITRO: PHAGOSOME PROCESSING STUDIED THROUGH OPSIN DEGRADATION IN ISOLATED BOVINE RETINAL PIGMENT EPITHELIUM CELLS DURING SHORT-TERM INCUBATION.

As discussed before the intact eye (intact RPE-retina complex) or the isolated eyecup are too complex a system to investigate the process of phagocytosis and its regulation on the level of the RPE. Rather, from the biochemical point of view, isolated RPE cells would be a much better substrate. This prompted us to investigate whether the short-term incubation system developed for isolated bovine RPE cells developed towards studies on retinoid metabolism (chapter 5) might be suitable to study phagocytosis as well. Hereto, we analyzed the kinetics of opsin digestion and the fragmentation pattern of endogenous opsin during short-term incubation using DIA and immuno-electroblotting.

RPE cells were isolated from cattle slaughtered throughout the light period and subjected to short-term incubation (0.5 to 6 hr). After several time intervals (0.5,1, 1.5, and 4 hr) cells were sampled and prepared for analysis with DIA (fig 7.8) or electro-immunoblotting (fig 7.9).

The pattern of opsin digestion nicely fitted into the profile observed in vivo (fig 7.8), indicating that the digestion kinetics were not affected by isolation and short-term incubation in vitro. Likewise, the fragmentation pattern and rate of digestion of intermediate fragments in the incubated cells was very similar to those observed in vivo (fig 7.9 vs fig 7.7). A 'background' population of intact opsin probably again represents predominantly rod outer segment contamination. No indication was obtained for large scale endocytosis of this opsin population during the time-frame of these experiments.

The similarity of the phagocytotic progress in vitro during short-term incubation and in vivo already demonstrates the potential of this system for studiesof phagocytosis. Further experiments are still needed to fully explore the merits of this system.

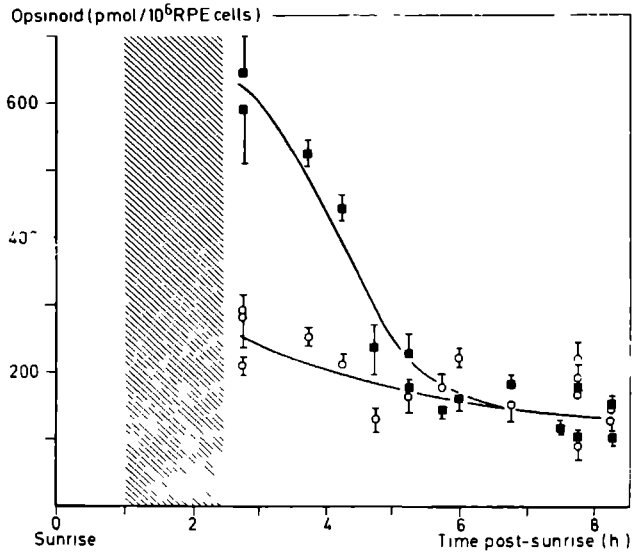


Fig. 7.8
Comparison of the course of opsin digestion during phagocytosis in bovine RPE in vivo and in vitro.

This combination of in vivo (cf fig 7.6) and in vitro data demonstrates that both data sets follow the same pattern. The opsin population in the incubated RPE cells are again determined by DIA. Open circles represent intact opsin. Filled squares represent total opsinoids. Isolated RPE cells were kept in short-term incubation, starting a 2.45, 3.45, 4.15, 6.00 7.45 hr post-sunrise, for 0.5, 1, 1.5 and 4 hr respectively and then immediately prepared for DIA. Data points represent 2 measurements on 1 sample. Error bar indicates standard deviation.

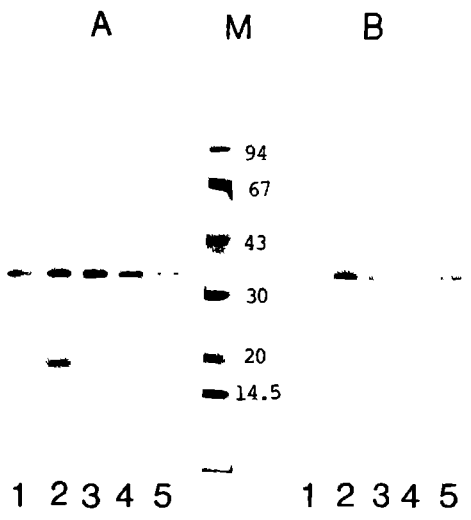


Fig. 7.9
Fragmentation pattern of opsin during continuing phagocytosis in RPE cells in vitro.

Samples of isolated bovine RPE cells (2 x 10^4 cells/lane) were er subjected to electro-immunoblotting aftvarious times in short-term incubation. The opsinoids were detected with Anti-N antiserum (dilution 500-fold): gel A; and Anti-C antibodies (dilution 200-fold): gel B. Cells were isolated 3.45 hr post-sunrise (lane 1) and incubated for 0.5 hr (lane 2), 1 hr (lane 3), 1.5 hr (lane 4) and 6 hr (lane 5). Preimmune sera gave no detectable reaction. M represents the molecular weight markers (MW x 10^{-3} kD).

7.4. CONCLUSIONS.

7.4.1. A NEW DIRECT ASSAY FOR THE PROGRESS OF PHAGOCYTOSIS IN VIVO AND IN VITRO: THE DIFFERENTIAL IMMUNO ASSAY (DIA).

The newly developed DIA allows direct quantification of opsin 'in statu digestandi'. This immediately demonstrates the fundamental difference with current assays for phagocytosis which are based on morphometry of phagosomal bodies. In comparison with the latter approach the DIA lists the following advantages: (i) local variations in the RPE cell population, which seriously affect the reproducibility of phagosome counting assays, are averaged out by assaying the total RPE cell population isolated from one or more eyes. Consequently, the number of individual assays can be reduced (compare fig 7.3 with fig 7.5, presenting the S.E. of 2 to 4 duplo estimations/data point and the S.D. of 2 determinations/data point respectively). (ii) The DIA presents a molecular picture of phagocytosis and simplifies discrimination between intact, largely extracellular, opsin and opsin 'in statu digestandi' to the presence or absence of 20 - 30 C-terminal residues of opsin. (iii) In the laborious sample preparation technique required for morphological analysis on the light- or electro- microscopic level, preparation of the RPE cell sample for DIA measurements is very simple and several samples can be handled simultaneously. Following resuspension in a suitable buffer and sonication, the cellular homogenate can be used directly in the inhibition assays. Due to the sensitivity of this type of assay, maximally 2×10^6 RPE cells are required to measure their content of opsin 'in statu digestandi'.

The principle of the DIA can be equally well applied to other protein modifications like proteolytic degradations or processing of precursors (post translational protein modification) of e.g. secretory proteins or membrane proteins. The only requirement is that monoclonal antibodies directed against the site to be modified, are available.

7.4.2. PHAGOCYTOSIS BY BOVINE RETINAL PIGMENT EPITHELIUM CELLS.

Bovine retinal tissues present several advantages in studying

phagocytosis by RPE. Bovine eyes are available in large quantities and in addition provide a large amount of retinal tissue allowing the isolation of relatively large amounts of intact RPE cells. In spite of these advantages, very little research has been carried out on phagocytosis in this species, probably due to the fact that it is not accessible to analysis of light/dark cycle shifts in a large population.

Our observations in cattle support the general accepted concept that the phagocytotic process in the mammalian RPE-retina complex follows a common circadian pattern (cf Besharse, 1982). (i) A burst of phagocytotic activity around the beginning of the light period (sunrise). (ii) The measured digestion rate for opsin in bovine RPE is sufficient to process its daily load within 5 hr. This is approximately the same time as required for phagosome processing in rat or opossum (ca 4 hr). (iii) The major ultrastructural transitions occurring during phagosome processing reported for rat, are observed in the bovine RPE cell as well (phagosome type I, II and III).

Finally, we have been able to demonstrate for the first time that the proposed relation between progress in opsin digestion and transitions in phagosome morphology indeed exists. The first ultrastuctural change in the phagosome, which appears to be induced by lysosome fusion, is accompanied by rapid initiation of proteolytic digestion. The second transition occurs when the digestion has reached a very advanced stage.

7.4.3. POTENTIAL OF ISOLATED RPE CELLS TO STUDY PHAGOCYTOSIS IN VITRO.

Investigation of the biochemistry and physiology of the process of phagocytosis on the level of the RPE cell requires separation of the RPE-retina complex. So far, eye-cup preparations, explants (small piece of RPE cells still attached to Bruchs membrane and choroidal tissue) or RPE-cell cultures have provided the tools to address this issue. The use of RPE cell cultures requires considerable caution, in view of dedifferentiation processes (chapter 4) with unknown consequences for the phagocytotic activities. The eyecup or explant preparations do not allow

directional biochemical analysis, due to the presence of large amounts of choroidal tissue. These problems would be avoided by using isolated RPE cells in a short-term incubation system. Dedifferentiation is not expected during these short incubation times. So far we have evaluated this system with respect to kinetics of digestion and pattern of fragmentation of opsin taken up by endocytosis in vivo. These parameters are not significantly different from those in vivo. We conclude that isolated RPE cells in short-term incubation provide an attractive alternative to investigate several aspects of the process of phagocytosis typical for RPE.

Chapter 8

SUMMARY AND GENERAL DISCUSSION.

Light reception, the primary basis for vision, requires proper functioning of the photoreceptor cells and in particularly of their outer segments, which contain the visual pigment rhodopsin. For proper maintenance of the photoreceptor function the retinal pigment epithelium (RPE) is indispensable. Major activities of the RPE in this intimate relationship between RPE and retina comprise metabolic manipulation of vitamin A (visual cycle) and phagocytosis of rod outer segment tips (outer segment turnover). Malfunctioning of the RPE or detachment of the retina from the RPE impairs these activities, which on a rapid time scale results in less efficient or blocked regeneration of the visual pigment, and on a longer time scale produces accumulation of membraneous debris between retina and RPE, which eventually leads to degeneration of the photoreceptor layer and thus to blindness.

Despite the general awareness of the vital contribution of the RPE to photoreceptor cell activity, fundamental knowledge on the physiology of the RPE cell is poor. Cellular cq subcellular *localization of metabolic conversions of vitamin A and* the regulation of its response to light or dark adaptation are still a matter of conjecture. In addition, several intriguing aspects of the role of the RPE in the circadian process of phagocytosis (trigger, response, control) are a complete mystery as well.

The scope of this investigation was to develop an experimental approach which would allow the study of RPE in vitro on a biochemical and on a cell-biological level. Such biochemical and cell-biological studies however require large amounts of relatively pure, intact cells. This requirement is not easily fulfilled since the retinal pigment epithelium forms a small layer of fragile cells between two complex adjacent tissues (retina and choroid) which complicates studies in vivo or in explant. The isolation of intact cells in high yield and purity is not simple either. The current methods (release of RPE cells from Bruchs membrane by means of brushing or application of proteolytic enzymes) are not satisfactory. Therefore our first task was to develop an alternative procedure which would allow isolation of pure, intact RPE cells in high yield.

Chapter 3 describes the development of a new isolation procedure for bovine retinal pigment epithelium cells, which is based on a 15

min-perfusion of the whole eye via the central ophthalmic artery with a cold, buffered isotonic salt solution free of divalent cations. The perfusion strongly reduces the extracellular Ca^{++} and Mg^{++} concentration and thus weakens the association of the retinal pigment epithelium cells with Bruchs membrane as well as the adhesion between retina and RPE. In addition the blood cells are flushed from the ocular vasculature. After careful removal of the retina, the RPE cells came off the Bruchs membrane easily and in large sheets upon applying gentle jets of buffer solution. This approach provides intact retinal pigment epithelium cells in high yield and purity (1 - 2 x 10^6 RPE cells/bovine eye). These cells show a high degree of viability (ca 80% of the cells exclude didansylcystine), high retention of cytoplasmic components (ca 85% retain cellular retinol binding protein) and excellent preservation of ultrastructural features. Hence, RPE cells isolated in this way could be very suitable for metabolic studies on e.g. vitamin A metabolism, phagocytosis and other biosynthetic or regulatory mechanisms. Such studies can in principle be performed with these freshly isolated cells but also with cells in short-term incubation or long-term cell culture. The potential of these bovine RPE cells for studies on vitamin A metabolism and phagocytosis was the further subject of investigation.

The limited availibility of freshly isolated RPE cells require a high frequency of cell isolation in order to fulfill the need for material for biochemical studies. Therefore it seemed more logical to opt for another approach: maintenance of RPE cells in culture (chapter 4). The advantages are obvious: large amounts of pure RPE cells in controlled environments, not influenced by components of retina or choroid and available at any time.

The potential of RPE cells in culture was evaluated according to morphological features and to elements, active in the vitamin A metabolism: the level of retinoid binding protein and the capacity to incorporate and metabolize retinol.

The isolated bovine RPE cells could be succesfully cultured at $37^{o}C$ in Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% foetal calf serum (FCS) in a humidified atmosphere of 5% CO_2. The cells exhibited a doubling time of 2 - 3 days and could be subcultured up to 6 times. Although many epithelial cells

preferentially grow on a collagen substrate, the bovine RPE cells appeared to be indifferent to whether the culture surface was coated (collagen type IV, polylysine, gelatin or foetal calf serum) or not.

Unfortunately, the cells in culture exhibited obvious signs of serious dedifferentiation. Morphological features like pigmentation, cell polarity and contact inhibition were completely lost. Furthermore, the level of cellular retinol binding protein (cRBP) and of cellular 11-cis retinoid binding protein (cRAlBP) was reduced to less than 10%. The ability to metabolize retinol declined precipitously as well. Since retinol is known to support epithelial differentiation, the culture medium was supplemented with retinol (0.4 µM, bound to BSA), in an attempt to prohibit the process of dedifferentiation. However, no significant improvement was observed with respect to morphology or level of cellular retinol binding protein. In spite of the fact that the isolated bovine RPE cells were easily cultured, we had to conclude that in *cell-culture these cells were not suitable for the investigation of* physiological functions of the RPF.

Since our scope was to investigate physiological functions of the RPE cell, we did not further explore RPE cells in culture. The loss of cellular polarity is very likely a major and perhaps primary cause of the dedifferentiation observed in cell culture. This problem might be overcome by an interesting recent technical development (compartimentalized incubation wells), which in particular seem to improve the conditions for epithelial cells. Hereto the cells are seeded on a semi-permeable membrane which would allow the RPE cells to take up nutrients through their basal membrane and transfer substances baso-apically. A major advantage of this approach is that the cellular polarity is maintained, which could very well reduce the dedifferentiation process. Such an alternative culture procedure therefore still deserves thorough experimental evaluation.

Chapter 5 describes an alternative approach to investigate the physiological properties of the RPE cells in vitro. A short-term incubation system was developed in which RPE cells remain viable for at least 8 hr. The energy charge and the retention of small cytoplasmic components, like nucleotides and cellular retinol

binding protein, were used as indicators for the cellular condition. We could demonstrate that the cells showed excellent survival in RPMI 1640 DM at 37°C under 95% O_2/5% CO_2. The energy charge rapidly increased during the first two hours and then leveled off to a level within the normal range of 0.8 to 0.95, which was maintained for at least 6 hr. The level of cRBP remained constant throughout the incubation. The total cellular adenine nucleotide level sharply increased during the first two hours and thereafter exhibited a slow monotonous increase.

In addition, chapter 5 describes the analysis of the retinoid status of bovine retinal pigment epithelium during short-term incubation up to 6 hr. The various reports in the literature on the total retinoid level of bovine RPE cells are in serious discrepancy and range from 1640 to 7500 pmol/10^6 cells. A similar degree of discrepancy exists with regard to the isomeric composition of the retinoids (see table 5.2) and the level of saturation of cRBP and cRAlBP (50 - 100%). The cause of these discrepancies are still a matter of speculation: light/dark history of the eyes, nutritional status, possible seasonal effects or isolation artefacts. It was very reassuring to notice that these figures showed little variation over a large number of our cell preparations: we arrive at an average total retinoid content of 1850 $\pm$ 120 per 10^6 RPE cells and an average saturation level of 45% for cRBP.

Short-term incubation of RPE cells did not affect the isomeric composition of the retinoid classes (retinol, retinaldehyde and retinylester) and was accompanied by a slight but not significant decrease of total retinoids, which predominantly (75%) originated in the retinylester fraction, while the remainder was due to decrease of retinol. This effect was independent of the light conditions. The reduction in the retinol level (ca 50%) was not due to leakage or secretion as the 'missing' retinol was not recovered in the incubation medium. Since retinoids are known to affect expression of genes and are involved in the biosynthesis of glycoproteins, metabolic processing of retinol for other purposes is a likely cause.

Remarkably, incubation in the light only affected the isomeric status (conversion of 11-cis to all-trans) of the retinylester fraction, while the level of 11-cis retinaldehyde, which is much more sensitive to photo-isomerization, remained constant. Possibly

the 11-cis retinaldehyde is maintained at the expense of 11-cis retinylester. This phenomenon might be related to the reduction in 11-cis retinylester level observed in frog RPE in vivo during light adaptation (Bridges, 1976).

The analytical approach described above does not give further insight in metabolic pathways relevant to the visual cycle. This important functional aspect was addressed by supplying the RPE cells with labeled retinol and by following label distribution over the various retinoid pools in time (chapter 6). Such an experimental approach allows detailed investigation of the metabolic routes of retinoids in RPE cells in vitro. Several kinetic parameters (retinol uptake, esterification, oxidation) correlate well with data obtained in vivo.

Purified all-trans retinol, carried by phosphatidylcholine vesicles, is rapidly taken up by RPE cells. Immediately after uptake, the retinol is further processed without complete equilibration with the endogenous retinol pool. This stems from the observation, that the final relative specific activity of the total retinol pool appears to be strongly dependent on the extracellular retinol supply, in spite of retinol fluxes of 5 to 40 fold the endogenous unlabeled retinol content. Retinol is predominantly converted into all-trans retinylester, in agreement with the general concept that the ester is the storage form. The maximal uptake and esterification rates observed would 'in vivo' terms allow complete processing of retinol liberated from a maximal rhodopsin bleach within 20 min. This is within the physiological range.

Only minor amounts of labeled all-trans retinaldehyde were recovered. Its formation exhibited a linear relation with incubation time and with the extracellular retinol concentration.

These studies provide the first positive indication that the RPE cell is able to effectuate isomerization of all-trans retinoid into 11-cis. The present evidence strongly suggests that this occurs at the level of retinol. At low extracellular retinol levels little labeled 11-cis retinol was recovered, but at higher levels the 11-cis retinol content increased rapidly and subsequently leveled off. Leveling of the 11-cis retinol content was accompanied by a remarkable increase in the levels of labeled 11-cis retinaldehyde

and labeled 11-cis retinylester. The calculated maximal isomerization rates and 11-cis oxidation rates are much to slow to carry the regeneration as occurring in vivo, but are probably underestimated. Regeneration of the fully bleached retina would now take at least 15 hr, whereas in vivo this is accomplished in 60 to 120 min. This discrepancy cannot yet be explained, but it is conceivable that in particular the all-trans into 11-cis isomerization and the formation of 11-cis retinaldehyde are key processes and under control of regulating factors, which have not yet been optimized in our incubation conditions.

Our finding, that all essential retinoid interconversion reactions in the visual cycle can be performed by the RPE, supports first of all the major role of this cell type in the visual cycle. The current model of the visual cycle locates the site of isomerization in the Müller cells. This is predominantly based (i) on the presence of cRAlBP in the Muller cells, where it was shown to carry both 11-cis retinol and 11-cis retinaldehyde and (ii) on the complete lack of any other evidence, which might just only give a hint to locate the isomerizing activity. Our findings require a reconsideration (i) of the contribution of the RPE on the one hand and Muller cells on the other hand to the regeneration process and (ii) of the concept that two visual cycles might coexist: a long visual cycle (retinol leaving the retina) and a short cycle (retinol does not leave the retina). The RPE cells would contribute to the long cycle and provide back-up support in chromophore isomerization during peak requirements (high bleaching levels). The Müller cells would carry the short visual cycle and should be able to cover the need for 11-cis retinaldehyde at low illumination levels. Our findings that the isomerizing activity in RPE has a relatively low maximal rate, but is coupled to a large storage capacity, support the concept of two complementary visual cycles. However the calculated rate represents a lower estimate due to suboptimal conditions (see above) and hence need further substantiation. The recent observation that the drug diaminophenoxypentane which inhibits rhodopsin regeneration in vivo probably by enhancing the back isomerization of 11-cis into all-trans, appears also to inhibit the formation of and to reduce the level of 11-cis retinylester (cf Bernstein and Rando, 1985) is very valuable. Application of such 'short-circuiting' compounds in

short-term incubation studies on RPE cells, could provide detailed information on the complex circuiting of the very isomerization pathway and consecutive reactions. Another interesting development regards the possible involvement of neurotransmitters in communication between photoreceptors and RPE (light dependent secretion of dopamine by photoreceptor cells; presence of dopaminergic D2 in RPE cells; Bruinink et al., 1986). It is very attractive to speculate that such communication lines regulate important processes in the RPE like production of 11-cis retinaldehyde or phagocytosis.

Chapter 7 addresses this other major task of the RPE in its maintenance of proper functioning of the photoreceptor layer: phagocytosis of the rod outer segment tips. This process follows a circadian rhythm and demonstrates high activity shortly after the beginning of the light period. In this chapter the relation is demonstrated between degradation of (rhod)opsin and processing of the phagosomal body by means of immunohistochemistry with antisera recognizing specific sites of (rhod)opsin. For this purpose monoclonal Anti-C antibodies (directed against the tail C-terminal residues of opsin) and a polyclonal Anti-N antiserum (directed towards the N-terminal part of opsin) were applied.

Immunohistochemical analysis of the eyecup with these antisera demonstrated that only the rod photoreceptor cell, and in particular its outer segment, and the phagosomal bodies in the RPE were labeled.

Phagosomes could be labeled both with Anti-C antibodies and with Anti-N antiserum. Quantifitation of labeled phagosomes by immuno fluorescence demonstrated a much more rapid loss of Anti-C reactivity than Anti-N reactivity during progress of phagocytosis. Hence, C-terminal digestion precedes degradation of the N-terminal part of opsin. By means of immunogold double-label immuno-electron microscopy we were able to correlate the cleavage of the C-terminal part of opsin with a very early event in the processing sequence of phagosomal bodies, occurring within 30 min after endocytosis.

This observation formed the basis for a new assay: the differential immunoassay, which allows to quantitate the phagocytotic degradation of (rhod)opsin in vivo or in vitro. This differential immunoassay (DIA) determines (i) intact (rhod)opsin

which represents (rhod)opsin outside the RPE cells and very shortly internalized (rhod)opsin by means of an inhibition ELISA with Anti-C antibodies, and (ii) total opsinoids (intact + fragmented) by means of an inhibition ELISA with Anti-N antiserum. Subtraction of Anti-C reactive opsin from Anti-N reactive opsinoids yields opsin 'in statu digestandi'.

Analysis of the kinetics of (rhod)opsin digestion as well as the fragmentation pattern of (rhod)opsin in vivo and in vitro, showed that the time course of degradation is not affected significantly by the isolation and incubation of RPE cells in vitro. This suggests that the developed incubation conditions also provide a reliable concept to study the process of phagocytosis in vitro.

The mechanisms which connect the process of phagocytosis to the internal clock, which sets the circadian rhythm, are still largely unknown. A variety of agents, applied to eyecup preparations of X. laevis, have been shown to affect this process, but the primary effect of these agents can not be localized in such a system. Use of isolated (mammalian) RPE cells in short-term incubation might be an attractive option to address the regulation of phagocytosis at the cellular level. Furthermore, very little is known on the processing of constituents of rod outer segment other than (rhod)opsin which are phagocytized by the RPE as well, like the highly unsaturated fatty acids or important constituents of the transduction chain like the G-protein and S-antigen. The fatty acids are very likely to be recycled since the retina tenaciously holds onto its highly unsaturated fatty acid level even upon dietary deprivation. A current hypothesis states that phagocytotic processing of S-antigen, which like rhodopsin is a potential effectuator of autoimmune uveitus (cf Gery et al., 1985; J.J. Schalken, personal communication), might be the link to the induction of this retinal disease. Such issues can be addressed very well in vitro using RPE cells in combination with the developed short-term incubation system.

In the investigation described in this thesis, we only addressed a few aspects of the activities of the retinal pigment epithelium, which are of tremendous importance for support and maintenance of proper functioning of the photoreceptor layer and consequently of vision. We have demonstrated the potential of the developed in

vitro short-term incubation system for RPE cells to study vitamin A metabolism and phagocytosis. It may be expected that investigation of RPE cells in vitro will effectively help to advance the insight in the broad spectrum of functions and activities of these challenging and fascinating cells.

SAMENVATTING.

RETINA PIGMENT EPITHEEL BESTUDEERD IN VITRO.

Licht detektie in het oog wordt uitgevoerd door hoog gespecialiseerde cellen in het netvlies: de kegeltjes en staafjes cellen, waarvan de zogenaamde buiten segmenten het lichtgevoelige pigment bevatten. In de staafjes buiten segmenten (ROS) is dit een eiwit, rhodopsine, dat in platte membraanzakjes ligt ingebed. De lichtgevoelige funktie van dit eiwit is gebaseerd op een ingebouwde groep, van vitamine A afgeleid, die onder licht isomeriseert. De buiten segmenten staan in nauw kontakt met retina pigment epitheel (RPE) cellen en deze cellen zijn essentieel voor het continu funktioneren van het netvlies. Belangrijke funkties van het RPE zijn het vitamine A metabolisme (met betrekking tot de visuele cyclus) en fagocytose van de uiteinden van ROS. Aantasting van het RPE of loslating van de retina, heeft belangrijke consequenties: de regeneratie van het visueel pigment wordt verstoord en of er zal een accumulatie van membraan-debris tussen RPE en retina optreden, waardoor uiteindelijk de fotoreceptor laag degenereert met als gevolg blindheid.

Ondanks deze wetenschap is er toch nog betrekkelijk weinig bekend over de fysiologie van de retina pigment epitheel cellen. De plaats waar belangrijke vitamin A omzettingen uitgevoerd worden en de regulatie daarvan zijn onbekend. Eveneens bestaan er nog vele onduidelijkheden over de rol van het retina pigment epitheel in de fagocytose van de ROS uiteinden, dat volgens een circadiaans ritme verloopt.

Met het hier beschreven onderzoek werd gepoogd een methodiek te ontwikkelen waardoor RPE cellen in vitro bestudeerd kunnen worden op biochemisch en cel-biologisch niveau. Voor dergelijke studies zijn grote hoeveelheden zuivere en intakte cellen nodig. Het feit dat het retina pigment epitheel een cellaag is van zeer fragiele cellen, ingesloten tussen twee complexe weefsels (retina en choroid), bemoeilijkt het voldoen aan deze voorwaarde aanzienlijk. Met huidige isolatie methodieken worden RPE cellen verzameld of door het mechanisch losmaken van de membraan van Bruchs (brushing) of door enzymatisch digestie. Geen van beide methodes levert cellen op die voldoen aan de gestelde eisen (zuiverheid en intaktheid).

Het ontwikkelen van een alternatieve isolatie procedure waarmee zuivere en intakte RPE cellen geisoleerd kunnen worden, was dan ook noodzakelijk voor het onderzoek van RPE in vitro.

In hoofdstuk 3 wordt de ontwikkeling van een nieuwe isolatie procedure voor retina pigment epitheel cellen uit runderogen beschreven. De methode is gebaseerd op een 15 minuten durende perfusie van het intakte oog via de centrale ophthalmische arterie met een ijskoude, gebufferde isotone zoutoplossing vrij van tweewaardige cationen. Door perfusie wordt de extracellulaire Ca^{++} en Mg^{++} concentratie sterk verlaagd met gevolg dat de hechting van het RPE aan de membraan van Bruchs aanzienlijk wordt verzwakt alsmede het kontakt tussen RPE en retina. Na voorzichtige verwijdering van de retina kan het RPE gemakkelijk in grote groepen (velden, sheets) met een isotone zoutoplossing worden 'losgesproeid'. Op deze manier kunnen intakte RPE cellen geisoleerd worden met hoge opbrengst en zuiverheid (1 -2 x 10^6 RPE cellen per runder oog). De intaktheid van de geisoleerde cellen blijkt uit de exclusie van 'viabiliteits kleurstoffen' (80% neemt geen didansyl cystine op), het vasthouden van een klein cytoplasmatisch eiwit (ca 85% van het cellulair retinol bindend eiwit) en uitstekende behoud van ultrastructuur. RPE cellen geisoleerd met de perfusie techniek bieden wellicht een geschikt uitgangspunt voor studies aan het vitamine A metabolisme, fagocytose en andere biosynthetische of regulatoire mechanismen. In de volgende hoofstukken zal de potentie van dit cel preparaat in studies aan het vitamine A metabolisme en fagocytose geevalueerd worden.

Vanwege de beperkte beschikbaarheid van vers geisoleerde cellen is, om voldoende materiaal voor biochemische analyse te verkrijgen, een frequente celisolatie vereist. Het leek daarom zinvol om RPE cellen in kweek te brengen De voordelen liggen voor de hand: grote hoeveelheden zuivere cellen in een gecontroleerde omgeving die niet beinvloed wordt door de componenten van de retina of choroid.

Cellen in kweek werden beoordeeld op morfologie en activiteiten met betrekking tot het vitamine A metabolisme: het gehalte aan retinoid bindende proteinen en de mogelijkheid tot opnemen en metaboliseren van retinol.

Geisoleerde RPE cellen kunnen gekweekt worden in Dulbecco's

Modified Essential Medium (DMEM) aangevuld met 10% Foetaal Kalfs Serum (FCS) in een vochtige atmosfeer van 5% CO_2. De cellen hadden een verdubbelings tijd van 2 tot 3 dagen en konden 6 keer worden overgezet in subcultures. Epitheliale cellen groeien vaak bij voorkeur op een collageen substraat, de RPE cellen uit rund bleken echter geen onderscheid te maken in een wel gecoat (collageen type IV, polylysine, gelatine of foetaal kalfs serum) of niet gecoat kweek oppervlak.

De cellen in kweek vertoonden in ernstige mate dedifferentiatie. Morfologische karakteristieken zoals pigmentatie en polariteit van de cellen alsmede kontakt inhibitie gingen volledig verloren. Hierbij komt dat het gehalte aan cellulair retinol bindend eiwit en het cellulaire 11-cis retinoid bindend protein tot beneden de 10% gedaald en ook de capaciteit om retinol om te metaboliseren was aanzienlijk verminderd. Gezien het feit dat retinol een rol speelt in differentiatie processen (vooral bij epitheliale cellen) werd gepoogd om dedifferentiatie te remmen door toevoeging van vitamine A (0.4 µM, BSA gebonden) aan het kweekmedium. Dit bleek geen significant effect te hebben op de morfologie of hoeveelheid cellulair retinol bindend eiwit. Uit deze bevindingen moesten we concluderen dat door de vergaande dedifferentiatie de cellen in kweek geen geschikt substraat vormen voor studies aan de fysiologische funkties van de RPE cellen.

In hoofstuk 5 wordt een alternatieve benadering voor in vitro studies aan retina pigment epitheel cellen uitgewerkt. Een systeem voor korte-duur incubaties werd ontwikkeld waarin de cellen viabel blijven voor tenminste 8 uur. De 'energy charge' en behoud van een kleine cytoplasmatisch komponenten zoals nucleotides en cellulair retinol bindend eiwit werden als parameters gebruikt ter beoordeling van de conditie van de cellen. Op deze manier waren we in staat aan te tonen dat de cellen goed overleven in RPMI 1640 DM bij 37°C in een vochtige atmosfeer van 95% O_2 en 5% CO_2. Onder deze condities neemt de 'energy charge' snel toe gedurende de eerste twee uur en bereikt een plateau binnen de normaal waarde van 0,8 - 0,95. Dit niveau kon worden gehandhaafd voor tenminste 6 uur. Ook het totale adenine nucleotide gehalte stijgt zeer sterk gedurende de eerste twee uur en vertoont daarna een langzame doch constante toename.

Eveneens in hoofdstuk 5 werd de retinoid status van het retina pigment epitheel gedurende de korte-duur incubaties geanalyseerd. In de literatuur bestaat geen overeenstemming: de totale hoeveelheid retinoid varieert van 1640 tot 7500 pmol/10^6 RPE cellen. Een vergelijkbare bestaat bestaat met betrekking tot de isomere samenstelling van de retinoiden (zie tabel 5.2) en het verzadigings gehalte van cellulair retinol bindend eiwit en cellulair 11-cis retinoid bindend protein (50 - 100%). Naar de oorzaken hiervan kan alleen gegist worden: licht/donker voorgeschiedenis, voeding, seizoens invloeden of isolatie artefacten. De variantie in deze getallen, gemeten over een lange periode en een groot aantal preparaten bleek klein te zijn voor het celpreparaat verkregen met de perfusie techniek: het gemiddeld gehalte aan totaal retinoid bedraagt 1850 $\pm$ 120 per 10^6 RPE cellen, het gemiddelde verzadigings percentage van het cellulair retinol bindend eiwit bedraagt 45%.

Korte-duur incubaties bleken geen effekt te hebben op de isomere samenstelling van de retinoid klassen (retinol, retinaldehyde en retinylester). Incubatie resulteerde in een lichte doch niet significante daling van het retinoid gehalte voornamenlijk (75%) in de retinylester fraktie, de rest door afname in retinol. Dit effect was onafhankelijk van de licht condities. Het verlies van retinol (ca 50%) werd niet veroorzaakt door lek of secretie want de 'vermiste' retinol werd niet terug gevonden in het inkubatie medium. Het is bekend dat retinoiden gen expressie beinvloeden en betrokken zijn in de biosynthese van glycoproteines, mogelijk word retinol ook in andere metabole processen dan de visuele cyclus gebruikt.

Incubatie in het licht vertoonde alleen effekt op de isomere samenstelling van de retinylester fraktie (conversie van 11-cis naar all-trans), terwijl het gehalte aan 11-cis retinaldehyde (veel gevoeliger voor foto-isomerisatie) niet veranderde. Mogelijk wordt het gehalte aan 11-cis retinaldehyde gehandhaafd ten koste van 11-cis retinylester. Mogelijk is dit fenomeen vergelijkbaar met de reductie van 11-cis retinylester gehalte in kikker RPE in vivo onder invloed van licht.

De bovenstaande benadering geeft vanwege de stabiele retinoid pools geen verder inzicht in de metabole pathways met betrekking

tot de visuele cyclus. Dit belangrijke aspect werd bestudeerd door middel van incubatie van de geisoleerde cellen met radioaktief gelabeld retinol en het volgen van de label verdeling over de retinoid pools in de tijd (hoofdstuk 6). Met een dergelijke benadering kunnen de metabole routes van retinol in het RPE bestudeerd kunnen worden in vitro. Verscheidene kinetische parameters (retinol opname, esterifikatie, oxidatie) komen overeen met in vivo gegevens.

Gezuiverd retinol, geincorporeerd in phosphatidylcholine vesicles, werd snel opgenomen door RPE cellen en onmiddelijk verder omgezet zonder opmenging met de endogene retinol pool. Dit blijkt uit de relatieve specifieke aktiviteit van de totale retinol pool die sterk afhankelijk blijkt te zijn van de buiten cellulaire retinol concentratie ondanks fluxes van 5 tot 40 keer groter dan de endogene niet gelabelde retinol pool. Retinol wordt hoofdzakelijk omgezet in retinylester, dit strookt met het concept dat retinylester de opslag vorm is. De maximale opname en esterificatie snelheid komen neer op de verwerking van een hoeveelheid retinol die vrij zou komen door een volledige bleking van het totaal aan rhodopsin, binnen 20 min!

Alleen kleine hoeveelheden gelabeld retinaldehyde werden gemeten. De vorming vertoonde een lineair verband met inkubatie tijd en toegevoegde retinol koncentratie.

Deze studies leveren de eerste aanwijzingen dat het retina pigment epitheel in staat is tot isomerisatie van retinoiden van de all-trans naar de 11-cis configuratie. De verkregen gegevens suggereren dat dit gebeurt op het niveau van de retinol. Bij lage extracellulaire retinol koncentraties word weinig 11-cis retinol teruggevonden, maar bij hogere koncentraties nam de hoeveelheid 11-cis retinol sterk toe en bereikte vervolgens een plateau. Het bereiken van dit plateau vertoonde een sterke samenhang met de opmerkelijke toename van gelabeld 11-cis retinaldehyde en gelabeld 11-cis retinylester. De berekende maximale isomerisatie en 11-cis oxidatie snelheden blijken veel te laag te zijn om regeneratie van het visueel pigment in vivo te kunnen verzorgen. Echter, deze waardes zijn echter waarschijnlijk onderschattingen. Onder deze condities zou de regeneratie van totaal rhodopsin ca 15 uur bedragen terwijl in vivo 60 tot 120 min voldoende zijn. Dit verschil kan op dit moment niet verklaard worden maar het lijkt

niet onwaarschijnlijk dat in het bijzonder de vorming van 11-cis retinaldehyde gereguleerd wordt door faktoren die wellicht niet voorkomen in het inkubatie systeem.

De bevindingen dat alle essentiele retinoid omzetting in het retina pigment epitheel kunnen worden uitgevoerd, onderstreept als eerste de belangrijke rol van het RPE in de visuele cyclus. Volgens het huidige model van de visuele cyclus vindt de isomerisatie aktiviteit in de Muller cellen plaats. Dit is vooral gebaseerd op (i) het vinden van cellulair 11-cis retinoid bindende eiwit, dat zowel 11-cis retinol als 11-cis retinaldehyde bevatte en (ii) de totale afwezigheid van de kleinste aanwijzing waar de isomerisatie plaats zou vinden. Op grond van onze bevindingen lijkt een herbeschouwing op zijn plaats van (i) de rol van het RPE in het regeneratie process aan de ene kant en de Muller cellen aan de andere kant en (ii) het concept van twee visuele cycli: een lange (retinol verlaat de retina) en een korte (retinol blijft in de retina). De RPE cellen zouden een rol hebben in de lange cyclus om regeneratie te ondersteunen na sterke bleking, de Muller cellen zouden, in de korte cyclus, snelle regeneratie onder lage belichtings omstandigheden verzorgen. De observatie dat de isomerisatie snelheid in het RPE relatief laag is maar gekoppeld is aan een hoge capaciteit (opslag van 11-cis retinylester) wijst in de richting van twee cycli.

In hoogstuk 7 komt een tweede belangrijk aspect van het retina pigment epitheel aan bod: fagocytose van de uiteinden van de staafjes buiten segmenten. Dit proces verloopt volgens een circadiaans ritme en vertoont hoge aktiviteit kort na het begin van de licht periode. In dit hoofdstuk wordt de relatie tussen degradatie van (rhod)opsine en cellulaire verwerking van het phagosoom aangetoond met behulp van immunohistochemie met antisera die specifieke sites van opsine herkennen. Voor dit doel werd gebruik gemaakt van monoklonale Anti-C antilichamen (gericht tegen de laatste C-terminale residuen van opsine) en een polyklonaal Anti-N antiserum (gericht tegen het N-terminale deel van opsine).

Immunohistochemische analyse aan oogcup preparaten toonde aan dat alleen de staafjes cellen, in het byzonder de staafjes buiten segmenten, en de phagosomen in het RPE gelabeld werden.

Phagosomen werden zowel met Anti-C als met Anti-N antisera

gelabeld. Kwantificering van de gelabeld phagosomen met immunofluorescentie liet een aanzienlijk snellere afname van Anti-C reaktiviteit dan Anti-N reaktiviteit zien tijdens phagocytose. Hieruit volgt dat digestie van het C-terminale deel voorafgaat aan degradatie van het N-terminale deel. Door middel van immuno elektronen microskopie hebben we de splitsing van de C-terminus van opsin kunnen correleren aan een zeer vroege stap tijdens de verwerking van phagosomen en die plaatsvindt binnen 30 min na incorporatie van het aankomend phagosoom in de RPE cel.

Deze observatie vormt de basis voor een nieuwe assay: de differentiele immunoassay waardoor de mogelijkheid ontstond om de phagocytotische degradatie van (rhod)opsine in vivo of in vitro te quantificeren. Met de differentiele assay wordt bepaald: (i) intact (rhod)opsine, dit is een maat voor (rhod)opsine buiten de cel en (rhod)opsine dat nog maar net is opgenomen, met een inhibitie ELISA met Anti-C antilichamen en (ii) totaal opsinoid (intakt + gefragmenteerd) met een inhibitie ELISA met Anti-N antiserum. Het verschil tussen beide opsinoid populaties geeft opsin 'in statu digestandi'.

Analyse van de digestie kinetiek alsmede het fragmentatie patroon van rhodopsin in vivo en in vitro, toonde aan dat het tijdsverloop van de opsine degradatie niet significant wordt beinvloed door de RPE isolatie en aansluitende in vitro incubatie. Het ontwikkelde systeem voor korte duur incubatie blijkt dus eveneens de mogelijkheid te bieden tot het bestuderen van phagocytose in vitro.

In dit onderzoek werden maar enkele aspecten behandeld van de aktiviteiten van het retina pigment epitheel, dat van groot belang is voor het goed functioneren van de fotoreceptor laag en dus van zien. We hebben kunnen aan tonen dat het ontwikkelde systeem voor korte-duur inkubaties de mogelijkheid bied tot het bestuderen van het vitamine A metabolisme en phagocytose. Het ligt in de lijn der verwachtingen dat in vitro studies zullen bijdragen aan het verkrijgen van een dieper inzicht in het brede spectrum van funkties en aktiviteiten van deze boeiende cellen.

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CURRICULUM VITAE.

Ad Timmers werd geboren op 17-09-1955 te Hamilton, Canada. Begin 1960 kwam hij naar Nederland en begon in 1967 met de middelbare school opleiding aan het Mgr. Zwijssen College te Veghel en sloot deze af in 1973 met het behalen van het diploma Atheneum B. Aansluitend werd begonnen met de studie Biologie aan de Universiteit van Nijmegen. Het behalen van het kandidaats examen in 1978 werd gevolgd door het doctoraal examen in 1981 met als hoofdvak Plantenfysiologie (Botanie) en als bijvakken Moleculaire Biologie en Biochemie.

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A rapid versatile microassay for cellular retinol-binding protein using Lipidex-1000 microcolumns

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Abstract A new, rapid and versatile microassay for cellular retinol binding protein has been developed based on separation of bound and free ligand by means of Lipidex 1000, a hydrophobic Sephadex derivative. This requires quantitative manipulation of retinol in aqueous solution. The tendency of retinol to adhere to glass and plastic surfaces was overcome by addition of the detergent Ammonyx LO, which yields a micellar dispersion. Detergent concentrations up to 10 mM did not interfere with binding of retinol to Lipidex-1000 or binding protein. The binding capacity of Lipidex-1000 was found to exceed 400 nmol of retinol per ml of gel. Retinal pigment epithelium (RPE) cells were used as a source for cRBP (cellular retinol-binding protein). The binding protein is saturated with ligand by incubation for 60 min at room temperature at concentrations of free retinol over 180 nM. Separation of protein bound retinol from free retinol is achieved via Lipidex-1000. protein-bound (specific and nonspecific) retinol is not retained and is eluted by buffer with the protein fraction. Free retinol is retained by Lipidex and is subsequently recovered by elution with methanol. Total recovery of ligand approaches 100%. Analysis time is about 4 hr for a maximum of ca. 50 samples. Nonspecific protein binding can be determined equally effectively either by incubation with 3 mM PCMBS or by addition of a 100-fold molar excess of nonlabeled retinol. The day-to-day reproducibility is within 5% and the detection limit is 7 μg of cRBP/liter using labeled retinol. With a 10 fold sacrifice in sensitivity, the assay can also be run in a nonradioactive mode using fluorescence detection. Estimation of the cRBP level in other tissues with this new assay gives values in the same range as reported elsewhere using RIA. The levels decrease in the order retinal pigment epithelium > > kidney > testis > > brain. Even the low tissue levels in brain (23 pmol/g of tissue) can be measured with the Lipidex microassay. The very high levels in RPE (68,000 pmol/g of tissue) probably reflect the important role of this tissue in furnishing and recycling retinol for the visual process. — **Timmers, A. M. M., D. A. H. M. van Groningen-Luyben, F. J. M. Daemen, and W. J. De Grip.** A rapid versatile microassay for cellular retinol-binding protein using Lipidex-1000 microcolumns. *J Lipid Res* 1986 **27:** 979–987

Supplementary key words cellular retinol-binding protein • retinal pigment epithelium • Lipidex 1000 • binding assay • retinol solubilization • separation of free and bound retinol

The retinoids, vitamin A and derivatives, are a class of essential compounds with diverse biological activity. *a)* They have profound effects on cell differentiation and proliferation in many tissues and hence have become important targets of research in dermatology and oncology (1). *b)* The aldehyde derivative of retinol is directly involved in vision (2). *c)* Vitamin A or its esters are essential for reproduction and vision. The latter two processes cannot be supported by retinoic acid (2, 3).

Utilization of vitamin A in the body is regulated at several sites (4). Transport of vitamin A, a very lipophilic compound, from liver to target tissue occurs through the serum retinol-binding protein (sRBP) (5). This protein is synthesized and secreted by the liver. Entry of vitamin A from the blood circulation into the cells is mediated by an sRBP receptor (6). At the cellular level retinoids are transported by a cellular retinol-binding protein (cRBP) or a cellular retinoic acid-binding protein (cRABP) (7). The latter two retinoid-binding proteins are very common to many tissues (8). Two other retinoid-binding proteins appear to be exclusively located in the retina: the cellular 11-*cis*-retinoid-binding protein (cRALBP) (9) and the interphotoreceptor matrix retinoid-binding protein (IRBP) (10, 11).

Currently the working mechanism of retinoids both at the cellular and at the molecular level is a matter of very active research. The selectivity of the retinol-binding proteins appears to be an important parameter in this con-

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Abbreviations: cRBP, cellular retinol binding protein; RPE, retinal pigment epithelium; sRBP, serum retinol binding protein; cRABP, cellular retinoic acid-binding protein; cRALBP, cellular 11 *cis* retinoid binding protein; IRBP, interphotoreceptor matrix retinoid binding protein.

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text The biological activity of a retinoid seems to be directly related to its affinity for the cellular retinoid-binding protein (12)

For the assay of retinoid-binding proteins, several procedures have been described binding assays, employing separation of free and bound ligand by sucrose gradient centrifugation (13, 14), on charcoal coated dextran (15), or by gel filtration (13, 16–18), and radioimmunoassays (19, 20), employing radiolabeled binding protein and specific antibodies

In the context of our studies on retinal pigment epithelium (21), the need for a rapid, simple, and sensitive retinoid-binding protein assay was felt Upon reviewing the existing methods, several disadvantages became obvious Sucrose gradient centrifugation and gel filtration assays are lengthy and laborious Even the recently introduced modification of the gel filtration approach—the much more rapid mini-gel column centrifugation procedure (22)—still requires laborious manipulation Separation of free and bound ligand with charcoal-coated dextran may result in considerable loss of protein (23) Radioimmunoassays are very sensitive, but require purified and labeled retinoid-binding proteins as well as monospecific antibodies elicited specifically against every different retinoid-binding protein to be assayed

Recently a reliable and rapid binding assay for the fatty acid-binding protein has been introduced (23), which is based on separation of the free from the protein bound hydrophobic ligand (palmitate) by means of Lipidex-1000, a hydrophobic Sephadex-G25 derivative containing 10% (w/w) alkyl groups Since this matrix has high affinity for hydrophobic compounds and has very low affinity for protein (23), its use could present a promising alternative for the retinoid-binding protein binding assays described so far After improving conditions for manipulation of retinol in aqueous environment (inclusion of detergent), we found that under optimal conditions Lipidex-1000 indeed has a high capacity to bind free retinoids This led to the development of a new binding assay for cellular retinol-binding protein, which is rapid, specific, and sensitive

MATERIALS AND METHODS

Materials

Lipidex-1000 was obtained from Packard Instrument Co, Inc (Downers Grove, IL) All-*trans* [11,12 di-^{3}H]retinol (sp act 55 Ci/mmol) was from Amersham (Amersham, UK), unlabeled all-*trans* retinol was from Eastman Kodak Co (Rochester, NY), Ammonyx LO was from Millmaster Onyx International (Fairfield, NJ), *p*-chloromercuribenzenesulfonic acid (PCMBS) monosodium salt was from Sigma Chemical Co (St Louis, MO), Con-A Sepharose was from Pharmacia AB (Uppsala, Sweden), and Aqua Luma Plus scintillation fluid was from Lumac 3M BV (Schaesberg, the Netherlands) All other reagents were of analytical grade Incubation buffer contained 10 mM KH_2PO_4, pH 7 4, containing either 0 06% Ammonyx LO, (RPE) or 0 2% Ammonyx LO (rat tissue) Elution buffer contained 10 mM KH_2PO_4, pH 7 4

As a source for cellular retinol binding protein (cRBP), retinal pigment epithelium cells were used (24, 25) They were isolated from bovine eyes obtained from the local slaughterhouse by the perfusion technique described previously (21) Their cytoplasmic proteins are released by sonication (30 sec, Branson B12 Sonifier with microtip) and, following centrifugation (30 min, 40,000 *g*, 4°C) the supernatant was used directly in the retinol-binding protein assay

Bovine serum, isolated from blood collected at the local slaughterhouse, was used as a source of serum retinoid-binding protein

Interphotoreceptor retinol binding protein (IRBP) was isolated from bovine retinas as described by Adler and Evans (26) the fraction released from Con-A Sepharose was directly used in the binding assay

Preparation of rat tissue extracts

Male rats (3 months, 150–200 g, fed ad libitum on Hope Farm rat pellets RNH) were killed by cervical dislocation and immediately perfused through the left ventricle with an isotonic Ca^{2+},Mg^{2+}-free salt solution containing 2 mM EDTA (21) Several organs were dissected and, after wet weight determination, the organs were homogenized in 10 mM KH_2PO_4, pH 7 4, in a Potter-Elvehjem tube with tight-fitting plunger The soluble proteins were extracted for 16 hr at 4°C The supernatants obtained by centrifugation (60 min at 10^5 *g*) were stored at −20°C and used directly for the assay of cRBP

A stock solution of all-*trans*-retinol in ethanol was stored under argon at −70°C The aqueous working solution was prepared fresh for every experiment by diluting the ethanol stock in incubation buffer to 800 nM retinol and addition of ^{3}H-labeled retinol (about 6×10^5 cpm/ml)

PCMBS was dissolved in incubation buffer in a concentration of 8 mM

Protein concentration was determined according to Lowry et al (27) using BSA for calibration

Preparation of Lipidex-1000 microcolumns

Glass Pasteur pipets were cut off at the beginning of the capillary part and at the other end leaving a total length of about 4 cm Then 200 μl of a suspension of Lipidex 1000 (diluted 1 1 with MeOH) was pipetted into the column using a glass bead (2–3 mm diameter) for support After the methanol had eluted, the microcolumn was washed three times with 0 7 ml of ice-cold elution buffer Routinely, every microcolumn was used only once However, repeated use is, in principle, possible Ready-to-use columns can be stored for at least 1 week at 4°C

Cellular retinol binding protein assay using Lipidex-1000

Assays were carried out in duplicate In order to determine the "total (specific + nonspecific) retinol binding" capacity in the protein fraction, the following mixture was incubated for 30–60 min at room temperature 160 μl of soluble protein extract in incubation buffer (0–200 μg of protein/ml), 80 μl of incubation buffer, and 80 μl of 800 nM [^{3}H]retinol In order to freeze the binding equilibrium, the mixture was then cooled in ice and kept at 0°C for 10 min The entire incubation was performed under dim red light (> 590 nm) in order to prevent photoisomerization of retinoids Separation of protein bound retinol from free retinol can be performed under fluorescent light Two hundred fifty μl of the cooled incubation mixture was carefully pipetted on top of a Lipidex 1000 microcolumn (100 μl of gel) and the eluate was collected The microcolumn was then washed with 250 μl and 500 μl of elution buffer, respectively Elution and washing takes about 20 min The three aqueous eluates were collected in a 20-ml scintillation vial This represents "the protein-bound retinol" fraction The "free retinol" fraction, which becomes bound to Lipidex-1000 during the procedure, was subsequently eluted with two volumes of 250 μl of methanol (5 min) and collected in a second scintillation vial In order to equalize the radiation quenching factors, 500 μl of methanol was added to the protein bound retinol fraction and 1 ml of elution buffer to the free retinol fraction Then, 10 ml of scintillation fluid was added to each vial and, after thorough mixing, the amount of ^{3}H label present was determined in a Philips liquid scintillation counter

The extent of "nonspecific binding" of retinol in the protein mixture was measured in parallel samples either by preincubation of the protein extract for 30 min at room temperature in the presence of 3 mM *p*-chloromercuri benzenesulfonic acid (PCMBS), a strong inhibitor of cellular retinol and retinoic acid binding proteins (cRBP and cRABP) (28), or by addition of a 100 fold molar excess of nonlabeled retinol to the incubation mixture simultaneously with the labeled all-*trans* retinol

Sucrose gradient assay

The assay of retinol binding protein via sucrose gradient centrifugation was performed as described by Wiggert and Chader (25) Briefly, 250 μl of cooled incubation mixture, prepared as for the Lipidex microassay, was layered on top of a 4 5-ml 5–20% (w/w) sucrose gradient prepared in 10 mM KH_2PO_4, pH 7 4 After centrifugation (16 hr, 220,000 *g*, 4°C) the bottom of the tube was punctured and fractions of five drops each were collected Each fraction was mixed with 5 ml of scintillation fluid and counted as described above Nonspecific binding was measured as described for the Lipidex microassay

Calculation of specific binding

Lipidex microassay Total protein binding (PT). Since the recovery in the eluates of the total label applied to Lipidex microcolumns is nearly quantitative (93–100%), total label (free + protein bound) was calculated by adding the radioactivity measured in the protein-bound fraction (PT) to the radioactivity measured in the free retinol fraction (FT) That percentage of total label, which represents total protein bound retinol (% PT), then becomes

$$\%PT = \frac{PT}{PT + FT} \times 100\%$$

Nonspecific protein binding (PN) The amount of nonspecific binding to the protein fraction was resolved either by performing the assay in the presence of excess non labeled all *trans* retinol or by preincubation with a specific inhibitor (PCMBS) Again, total label was calculated by adding the radioactivity in the protein bound fraction (PN) to that in the free retinol fraction (FN) The percentage of nonspecific binding (% PN) was then

$$\%PN = \frac{PN}{PN + FN} \times 100\%$$

Specific protein binding (PS) PN + FN should equal PI + FT The amount of retinol specifically bound to binding protein in the 250 μl reaction mixture (PS) was then calculated as follows

$$PS = (\%\ PT - \%\ PN) \times 50\ pmol$$

Sucrose gradient assay The calculation of the specific binding of retinol as obtained by sucrose gradient centrifugation was also done by subtracting the nonspecific binding from the total binding

cRBP (*M*, ca 15 kD) migrates with a 2 S-sedimentation constant Hence, those fractions in the 2 S region, where the label density was higher in the assay for total binding than in the corresponding assay for nonspecific binding were considered to represent cRBP The radioactivity in these fractions was added together and represented total 2 S-bound label (PTS)

Likewise, the sum of the radioactivity in the corresponding 2 S fractions of the nonspecific binding assay was calculated and represented nonspecific 2 S binding (PNS) These figures were normalized into percentages of total label layered on the gradients, yielding % PTS and % PNS The specific binding in 250 μl of reaction mixture (PSS) was then calculated as follows

$$PSS = (\%\ PTS - \%\ PNS) \times 50\ pmol$$

RESULTS AND DISCUSSION

Retinol in aqueous media

Retinol is highly insoluble and rather unstable in aqueous solution. Consequently, it demonstrates a strong tendency in aqueous environment to aggregate and/or to adhere to whatever glass or plastic surfaces are available (29). This property makes it impossible to handle retinol quantitatively, and does not allow a calculation of the actual concentration of retinol in aqueous media from the amount added. These physical characteristics render assays of retinol-binding proteins based on binding of retinol troublesome and less reliable.

In order to overcome this disadvantage, we investigated whether the addition of detergents, which are routinely used for the solubilization of hydrophobic compounds in aqueous media, might also be suitable for aqueous dispersion of retinol. **Table 1** demonstrates that solubilization of retinol in aqueous media using low concentrations of detergent does indeed strongly suppress the tendency of retinol to adhere to glass and polyethylene surfaces. The detergent Ammonyx LO was selected for routine use in this respect since it is a commercially available, reasonably cheap, mild, and well-defined nonionic detergent. In the presence of at least 3 mM Ammonyx LO (0.06%), which is well above its critical micellar concentration of 1 mM (30), retinol appears to be completely in micellar form and behaves as a stable aqueous dispersion. Concentrations up to 0.5 mM retinol are easily attained. As deduced from spectral measurements, retinol solubilized in incubation buffer is stable for at least 4 hr at room temperature.

Retinol binding by Lipidex-1000

In order to explore the suitability of Lipidex-1000 in a binding assay for retinol-binding proteins, its capacity to bind retinol dissolved in Ammonyx LO must first be analyzed. This is performed by applying 250-μl aliquots of solutions with increasing retinol concentration in incubation buffer (0.06% Ammonyx LO) to 100 μl of Lipidex-1000 gel. Rapid and complete extraction of retinol out of its micellar solution could be achieved and no saturation of binding was observed up to the highest concentration tested (160 μM). Hence, the binding capacity of Lipidex-1000 for retinol amounts to at least 40 nmol of retinol per 100 μl of gel and easily satisfies the requirements for a binding assay for retinol-binding proteins. Higher detergent concentrations were also investigated. Ammonyx LO concentrations up to 0.5% (25 mM) did not affect the binding capacity of Lipidex-1000 for retinol.

A small percentage of leakage through the Lipidex microcolumn was observed, which appears to have been due to slow degradation of [^{3}H]retinol. This percentage was low when a fresh batch was used ($\leq$ 3%) but increased up to 10% in 6 months. The amount of protein bound retinol (total and nonspecific binding) was corrected for this leakage.

Source of cBRP

Retinal pigment epithelium was selected as a source for the cellular retinol binding protein since that tissue contains high amounts of this binding protein (24, 25) and we have developed a reliable procedure to isolate these cells in high yield and high purity (21).

Binding assay

Several conditions have to be fulfilled in order to allow reliable and quantitative determination of receptors or binding proteins by means of a binding assay. *1)* Separation of protein-bound ligand and free ligand must be achieved. *2)* Quantitation of binding protein requires saturation of the binding proteins with added ligand. Hence, incubation should be continued until a dynamic equilibrium between receptor and ligand has been attained and the concentration of added ligand should be sufficient to saturate the binding protein. *3)* Reliable discrimination

TABLE 1 Typical recovery of retinol in aqueous phase after 30 min incubation of 1 μm retinol at room temperature in some common vessel materials

Vessel Material	Recovery of Retinol (%)		
	Buffer Only	Aqueous Phase[a]	
		+ Nonylglucose (3 mM)[b]	+ Ammonyx LO (3 mM)
Polystyrene	13	35	85
Glass	30	80	96
Polyethylene	12	90	98

[a] Aqueous phase 10 mM KH_2PO_4, pH 7.4.
[b] Detergent concentration was below critical micellar concentration (6.5 mM, ref. 30).
[c] Incubation buffer detergent concentration was above critical micellar concentration 1 mM ref. 30).

between specific binding protein-dependent and nonspecific binding of the ligand should be accomplished

The binding assay for retinol binding protein using Lipidex-1000 was developed along these lines

The feasibility of separating protein-bound and free retinol with Lipidex-1000 was investigated by applying incubation mixtures containing cRBP and labeled retinol to the Lipidex microcolums Subsequently the columns were eluted as described under Methods The label not retained by the Lipidex represents protein bound retinol **Fig. 1** shows that a certain fraction of the retinol elutes in the "protein-bound retinol fraction" It further shows that a linear relation exists between the amount of total label eluting with the protein fraction and the amount of protein applied to the column We conclude that, in agreement with the results described for the fatty acid-binding protein (23), separation of protein-bound and free retinol can be achieved by means of Lipidex-1000

In order to determine the time to binding equilibrium during incubation of retinol with the binding protein at room temperature, the incubation time was varied from 5 min to 2 hr The amount of protein-bound retinol was measured as described under Methods Maximal binding was reached after 15–30 min of incubation at room temperature and remained constant for the next 2 hr (data not shown) In order to ascertain that equilibrium conditions are reached, incubation times of 60 min at room temperature were applied routinely

Saturation of binding is achieved when the amount of specifically protein-bound retinol levels off to a constant level upon increasing the total amount of retinol in the incubation mixture at a fixed binding protein concentration This permits selection of a retinol concentration where, rather independent of the amount of protein present in the assay, the amount of specifically bound retinol is a quantitative measure for the amount of binding protein present This aspect was addressed by incubating cell supernatant with a fixed amount of protein in the presence of increasing retinol concentrations for 1 hr at room temperature Subsequently, free and protein bound retinol were separated over Lipidex 1000 microcolumns as described above The amount of specifically protein-bound retinol first increased with increasing free retinol concentration, but then leveled off at concentrations over 180 nM (**Fig. 2**) Hence, a retinol concentration of 200 nM was used routinely in the binding assay This concentration corresponds to 50 pmol of retinol per 250 μl sample, which is still far below the binding capacity of the amount of Lipidex 1000 gel used (40 nmol) Fig 1 already confirmed that under these conditions a linear relation exists between amount of protein-bound retinol and protein concentration Hence, saturation and thus quantitation of binding protein can be achieved at 200 nM retinol provided that total retinol binding does not exceed 10% of retinol added

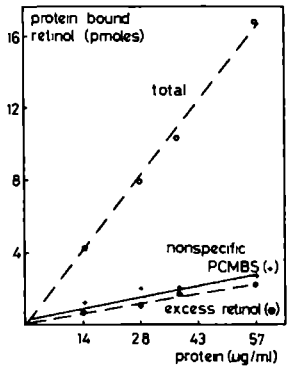

Fig 1 Separation of free and protein bound retinol by means of Lipidex 1000 and determination of nonspecific retinol binding in protein fractions A cRBP preparation (15–60 μg/ml) was incubated with 200 nM ^{3}H labeled retinol for 60 min at room temperature Total protein bound retinol was determined by the Lipidex microassay as described in Methods The total protein bound retinol is plotted versus protein concentration Nonspecific protein bound retinol was determined by addition of 100 fold excess of nonlabeled retinol (● ● ●) and by preincubation with 3 mM PCMBS for 30 min at room temperature (+ + +)

The last condition to be investigated is whether one can easily discriminate between specific and nonspecific binding of retinol The established method for determination of nonspecific binding in binding assays consists of incubating the binding protein under normal assay conditions, but thereby including a 100 fold excess of non labeled ligand We have used the same approach by incubating the cRBP preparation in the presence of 200 nM [^{3}H]retinol and additional nonlabeled retinol (20 μM) It is evident from Fig 1 that the amount of label eluting with the protein fraction is strongly reduced by incubation in the presence of a 100-fold excess of non labeled retinol An independent approach confirmed that the latter curve represents nonspecific binding It has been reported that retinol binding to the cellular retinol-binding protein is very sensitive to millimolar concentrations of PCMBS (28) Hence, treatment with PCMBS might be a suitable way to inhibit the specific binding of retinol to cRBP and thus to monitor nonspecific binding Upon preincubation of the cRBP preparation with 3 mM PCMBS for 0 5 hr at room temperature, a binding curve was obtained which indeed nearly coincided with the one obtained in the presence of excess nonlabeled retinol (Fig 1) Although different mechanisms are involved, PCMBS and excess nonlabeled retinol appear to be equally effective in monitoring nonspecific binding of retinol Since the high concentrations of retinol required might be undesirable under certain conditions, nonspecific binding was further routinely determined by preincubation with 3 mM PCMBS This concentration of

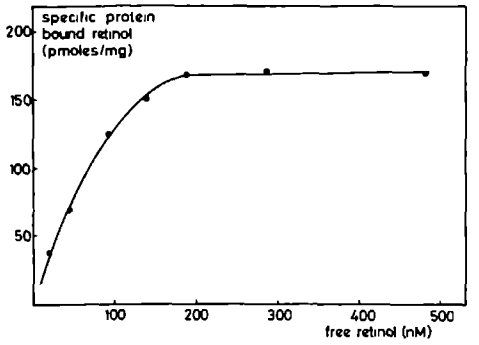

Fig 2 *Saturation of cellular retinol binding protein with all trans* retinol In this typical experiment, a cRBP preparation (57 μg/ml) was incubated with increasing retinol concentrations (25–500 nM) for 60 min at room temperature Parallel samples were preincubated with 3 mM PCMBS to determine nonspecific binding Specific binding was calculated and plotted versus free retinol concentrations The amount of specific protein bound retinol initially increased with increasing free retinol concentrations, but then leveled off at 180 nM retinol

PCMBS did not affect the binding capacity of Lipidex-1000 for retinol (not shown)

In conclusion, the Lipidex microassay unequivocally satisfies all requirements set for a reliable binding assay

Reproducibility and sensitivity

The Lipidex microassay had to be evaluated further with respect to reproducibility and sensitivity The day-to-day reproducibility was evaluated by assaying the same cell supernatant with respect to binding activity at intervals of several days The binding activity was thereby defined as the amount of specific bound retinol (total bound minus nonspecific bound as determined with PCMBS) per mg of protein Plots of the amount of specific bound retinol versus protein concentration (**Fig. 3**) show that the curves obtained over a 7-day period coincided within the experimental error The respective binding activities ± SD were calculated by linear regression (on five samples in duplicate per assay), and indeed did not change significantly over the various measurements Similar results have since been obtained repeatedly, which demonstrates that the assay allows very reproducible measurements

The detection limit of the assay, defined as the lowest binding protein concentration that shows a significant difference between total and nonspecific binding, was determined with the Student's *t*-test on data again obtained with RPE supernatants For this typical cellular retinol binding protein preparation, a detection limit of 0 5 pmol of retinol/ml was calculated ($P < 0\ 05$) With a molecular weight of 15 kD for the cellular retinol-binding protein (8) and a binding stoichiometry of 1 to 1, a retinol concentration of 0 5 pmol/ml corresponds to 7 μg/l of cellular retinol-binding protein Thanks to the fact that PCMBS can be used to measure nonspecific binding, the assay can be performed equally well with nonlabeled retinol, using fluorescence detection (λ_{ex} = 330 nm emission peaks at 480 nm) Under these conditions, the assay becomes about tenfold less sensitive (tenfold higher detection limit), but no special equipment for radioactive work and scintillation counting is required

Comparison with other assays

In the evaluation of a new assay, there should be a comparison with the existing procedures for the determination of cellular retinol-binding protein The approach based on sucrose gradient centrifugation has been employed most extensively, and has also been used by Miller et al (22) for comparison with a radioimmunoassay (RIA) and their improved gel filtration method Hence, we compared the performance of the sucrose gradient centrifugation and the Lipidex 1000 microcolumn procedure in the assay of RPE cell supernatant Using the same typical RPE preparation, we measured a binding activity of 280 pmol of retinol/mg of protein with the Lipidex microassay and a binding activity of 200 pmol of retinol/mg of protein with the sucrose gradient procedure, i e , about 71% of the Lipidex microassay This lower value may originate

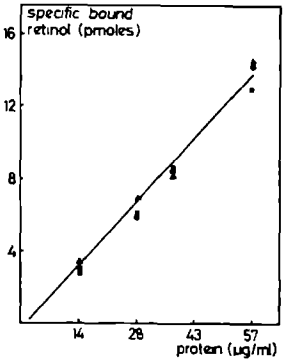

Fig 3 Reproducibility of the Lipidex microassay A dilution range of a cRBP preparation was assayed on 3 separate days (symbols representing days 1, 2 and 7 are ▲, ■, and ● respectively) Nonspecific binding was determined by preincubation with PCMBS Specific binding of retinol was calculated and plotted versus protein concentration The curve obtained by linear regression analysis of all data points yielded an average binding activity of 231 ± 9 pmol of retinol/mg of protein Similar analysis of the separate data points yielded values of 238 ± 18, 223 ± 12 and 234 ± 10 pmol of retinol/mg of protein at days 1, 2 and 7, respectively These values are not significantly different and illustrate the reproducibility of the assay

in the long separation times required by the sucrose gradient Miller et al (22) reported that under their conditions the sucrose gradient procedure yielded values of about 70% of the minicolumn assay and of about 60% of those values obtained by RIA Since the conditions for the sucrose gradient procedure used as a reference may not be identical for the two laboratories and its reproducibility is not as good as that of the other assays, this comparison only allows general conclusions Nevertheless, it is obvious that the Lipidex microassay may yield values comparable to gel filtration and RIA

In order to test the performance of the Lipidex microassay on tissues with lower cRBP content, we assayed several rat tissues for which cRBP levels have been reported in literature (19, 31, 32) We selected three tissues with relatively high, intermediate, and low cRBP levels, i e , kidney, testis, and brain, respectively The results are given in **Table 2.** It is evident that the tissue levels correlate very well with the literature values The relatively large variance is mainly due to biological variation This probably also explains the relatively large variation between data obtained by the various radioimmunoassays In one case we even measured a large difference between two kidneys from the same animal (1280 and 2420 pmol/g of tissue, respectively)

The lower cRBP content in these tissues implies that the protein concentration in the incubation mixture must be increased (500–1000 μg/ml) relative to RPE (≤ 200 μg/ml) Under these conditions, insufficient detergent was available at a total concentration of 3 mM (0 06%) to achieve complete solubilization of retinol and, therefore, the total detergent concentration in the incubation mixture was raised to 10 mM (0 2%) leaving the other conditions unchanged The effect of the detergent concentration on the assay performance was tested on the RPE extract Detergent concentrations in the incubation mixture up to 13 mM did not interfere with the assay At higher concentrations, a concentration-dependent decrease in binding activity was observed

Discrimination between the various retinoid binding proteins

Finally, the question was addressed as to whether the Lipidex microassay as described was selective for cRBP in the presence of other retinoid-binding proteins

Considering cRALBP (cellular 11-*cis*-retinoid binding protein) and cRABP, it can be safely stated that the high specificity of these binding proteins for their respective ligands (12) guarantees that they will bind only insignificant amounts of all-*trans* retinol and will not interfere with the cRBP assay Indeed, while RPE contains a high level of cRALBP (700 pmol/eye (33)), the value we measured with the Lipidex microassay for cRBP (550 ± 50 pmol/eye) was in excellent agreement with levels inferred from biochemical analysis (500 pmol/eye (33)) Similarly, the cRBP levels measured in the various rat tissues which contain significant amounts of cRABP (19, 32) agreed well with the literature values obtained by RIA

Rather, one might expect interference of the other retinol-binding proteins described so far (cRBP-II in small intestine (34), the extracellular sRBP in blood and IRBP in the interphotoreceptor matrix) cRBP II has only been detected in small intestine and its biochemical parameters (Kd, ligand specificity) have not yet been reported If they turn out to be very similar to those of cRBP, a binding assay like the Lipidex microassay will not be able to distinguish between cRBP and cRBP-II and will measure the total Interference with the extracellular RBPs, which might contaminate certain tissue extracts, is easily eliminated An evaluation of the performance of the Lipidex microassay on serum (sRBP and serum albumin) and purified IRBP showed that binding of retinol to these extracellular RBPs is not inhibited by 3 mM PCMBS (data not shown) Hence, by preincubation with PCMBS, *only* the cellular retinol binding protein is inactivated and will contribute to the specific binding activity determined by subtracting PCMBS-insensitive binding from total binding

TABLE 2 Levels of cRBP in rat tissues Comparison of the Lipidex 1000 microcolumn assay with literature values obtained by RIA

Species	Tissue	RIA Adachi et al (Ref 31)	RIA Kato et al (Ref 32)	RIA Ong et al (Ref 19)	Lipidex 1000 Assay
		pmol/g wet weight ± SD			
Bovine	RPE				68 000 ± 6 200 (3)*
Rat	Kidney	3980 ± 1000	1631 ± 175	513 ± 16	1,702 ± 556 (4)
Rat	Testes	1270 ± 260	1113 ± 300	264 ± 23	394 ± 189 (2)
Rat	Brain	270 ± 150	275 ± 69	27 ± 2 2	23 ± 7 (1)

*Number in parentheses is the number of different tissue samples Assays on the same sample were performed two to four times over a time interval of several months

In pilot studies, we further investigated whether Lipidex-1000 can be applied to assay the other cellular retinoid binding proteins, viz the 11-*cis*-retinoid-binding protein (cRALBP) and cellular retinoic acid binding protein (cRABP), respectively One of the major questions will be whether Lipidex-1000 has sufficient affinity for their ligands to allow separation of protein-bound and free ligand Titration of Lipidex-1000 with ligands dissolved in incubation buffer (0 06% Ammonyx LO) have not yet established upper limits, but show complete extraction both of retinoic acid and of 11-*cis*-retinaldehyde up to at least 12 5 nmol per 100 μl per gel We conclude that the Lipidex microassay can, in principle, be adapted to the other cellular binding proteins

CONCLUSIONS

The Lipidex microassay that we have developed satisfies all criteria for a reliable binding assay for cellular retinol-binding proteins It is simple, reproducible, yields a linear relation between protein concentration and retinol binding over a wide concentration range (2-1000 μg/ml), and it conforms to existing procedures In addition, it has several definite advantages over the existing procedures (radioimmunoassay or free/bound ligand separation by sucrose-gradient centrifugation, gel filtration, or charcoal adsorption) *1)* It is rapid and requires relatively little equipment and manipulation Manually, up to 50 samples can be easily handled simultaneously, with a processing time of ca 4-5 hr (excluding scintillation counting, which can be performed overnight) The procedure lends itself well to automation *2)* With some sacrifice in sensitivity (ca tenfold decrease), the procedure can be run equally well in a nonradioactive mode, and then requires only a simple fluorometer for quantitative measurements *3)* The Lipidex microassay can easily be applied to several tissues and, in contrast to immunoassays, no highly specific and/or rapidly decaying reagents are required *4)* In principle, the Lipidex microassay is very versatile Programming onto the other cellular retinoid-binding proteins (cRALBP, cRABP) should be easy Their ligands (11-*cis*-retinaldehyde and retinoic acid, respectively) can be extracted from the incubation mixture with the same efficiency as retinol, when not bound to protein

We thank Dr J Rodrigues de Miranda for critical reading of the manuscript and Miss A van Alst for her excellent text editing This investigation was supported by the Netherlands Foundation for the advancement of Basic Research (ZWO)

Manuscript received 5 August 1985

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STELLINGEN

I

Door geen rekening te houden met de slechte oplosbaarheid van retinoiden in waterig milieu zijn de gerapporteerde waarden voor de dissociatiekonstante van retinoid bindende eiwitten uiterst dubieus.

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U. Cogan, M. Koppelman, S. Mokady and M. Shinitzky (1976). Eur. J. Biochem., 65: 71-78 D.E. Ong and F. Chytil (1978). J. Biol. Chem., 253: 828-832

II

De door Longstaff et al. gepubliceerde gegevens wettigen niet de conclusie, dat deprotonering van de Schiffse base in rhodopsine in het intermediair stadium Meta II noodzakelijk is voor de activering van transducine.

C. Longstaff, R.D. Calhoon and R.R. Rando (1986). Proc. Natl. Acad. Sci. USA, 83: 4209-4218

III

De veronderstelling dat er een universeel GTP-bindend eiwit in het Ca^{++} messenger systeem bestaat, behoeft herziening vanwege verschillen in gevoeligheid van dit messenger systeem voor pertussis toxine in verschillende weefsels.

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IV

Het gebruik van artificieel substraat bij onderzoek aan fagocytose door retina pigment epitheel is biologisch weinig relevant.

R.B. Edwards and P.M. Flaherty (1986). J. Cell. Physiol., 127: 293-296

V

Op basis van de gepresenteerde resultaten zijn andere verklaringen mogelijk dan dat de isomerisatie van vitamine A tijdens de regeneratie van rhodopsine verloopt volgens een niet stereospecifieke route.

P.S. Bernstein, J.R. Lichtman and R.R. Rando (1985). Biochemistry, 24· 487-492

VI

Het benoemen van eiwitten als 'retina specifiek' getuigt, wanneer deze eiwitten inmiddels zijn gelokaliseerd in ander weefsel, op zijn minst van een onzorgvuldig taalgebruik.

I. Gery, M. Mochizuki and R.B. Nussenblatt (1985). Progr. Retinal Res., 5: 75-109

VII

Het verdient aanbeveling patienten met retina loslatingen en behandeling daarvan te testen op autoimmuniteit tegen de alle thans bekende uveitogene eiwitten.

VIII

De stijging van het aantal mensen dat gebruik maakt van psycho-sociale trainingen staat in direkt verband met de toenemende bewustwording van eenzaamheid.

IX

De technologische ontwikkelingen in de medische wetenschap bedreigen het humane karakter van de eed van Hippocrates.

X

Het vol vuur strijden tegen de Apartheid mag niet ontaarden in een MAKROscopische brandstapel.

Nijmegen, 25 februari 1987 Ad Timmers